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EVALUATING THE EFFECT OF NON-THERMAL PROCESSING AND ENZYMATIC HYDROLYSIS IN MODULATING THE ANTIOXIDANT ACTIVITY OF NEBRASKAN GREAT NORTHERN BEANS

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Great Northern Beans (GNB), a major pulse crop of Nebraska, are a rich source of dietary proteins and has the potential to release peptides with beneficial biological properties upon enzymatic hydrolysis. Thermal processing, such as boiling, is the most common way to process beans; it makes the beans palatable and also improves the accessibility of the proteolytic enzymes. Heat treatments destroy the naturally present essential bioactive components in beans like phytochemicals and could be prevented using an alternative non-thermal process. High Pressure processing (HPP), a non-thermal process and the most used alternative to thermal processing, can influence the storage proteins of beans and can modulate the enzymes' accessibility to cleave proteins. Thus, the objective of the present study was to evaluate the efficacy of HPP of 600 MPa for 5 mins to produce antioxidant peptides from GNBs compared to boiling after alcalase-hydrolysis. Our results suggest that the degree of hydrolysis (DH) of the cooked beans were significantly higher compared to the HPP-treated beans, with no difference in the total peptide content. The oxygen radical absorbance capacity (ORAC) of the alcalase-hydrolysate of cooked beans have a higher antioxidant activity (370.9 ± 43.8 μmol TEAC/g) compared to that of HPP-treated beans (285.3 ± 53.2 μmol TEAC/g) where,

TEAC is the Trolox equivalent antioxidant capacity. Therefore, the study concludes that boiling is a more efficient process than HPP to produce peptides with antioxidant activity but HPP is found to be effective in retaining the stability of phenolic compounds present in the beans.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION

Dry edible beans refer to the 'pods' and belong to the class of pulses. Pulses are legumes that are high in proteins, low in fats, high in dietary fibers, and have health-beneficial roles in the gastrointestinal digestion, regulates cholesterol absorption, and maintains the energy level in the biological systems (FAO, 2016). Dry edible beans (*Phaseolus vulgaris L.*) are widely consumed as a component in the human diet as it is an essential source of dietary proteins among other plant sources (Ganesan and Xu, 2017). For the past few decades, dry edible beans have been extensively studied as a promising functional food source as it is rich in proteins, phytochemicals, minerals, vitamins, and dietary fibers (Reynoso-Camacho et al., 2006).

Phytochemicals are naturally present in beans and can exhibit health-beneficial biological properties like antioxidant and anti-inflammatory activities (Cardador-Martínez et al., 2002; Wu et al., 2004; Heimler et al., 2005; García-Lafuente et al., 2014). Most of the phytochemicals, primarily the flavonoids are found in the seed coat of the beans (Aquino-Bolaños et al., 2016). Some of the flavonoids, such as anthocyanins, and flavonols like quercetin from beans, were reported to show high antioxidant activity (Beninger and Hosfield., 2003). Beans are also a rich source of phenolic acids such as p-coumaric acid and ferulic acid; phenolic acids are also well known for their antioxidant activities (Kim and Park., 2019). Along with phytochemicals, the high protein content in beans compared to other plant-based foods, proves beans to be a potential source to produce peptides with biological activities (Oseguera Toledo et al., 2016). Earlier studies have demonstrated that controlled enzymatic hydrolysis of bean proteins can produce peptides that can exhibit antioxidant activity by scavenging the free radical formation (Karas et al., 2014).

However, to produce bioactive peptides from whole beans, a heat treatment is required to denature the native proteins, enhancing the accessibility of proteolytic enzymes to facilitate the hydrolysis of the peptide bonds at specific cleavage sites. The heat treatment has been reported to degrade the phenolic compounds and flavonoids naturally found in beans (Ketharin et al., 2019). The destruction of the flavonoids due to heat treatments, can reduce the antioxidant properties of the beans (Xu and Chang, 2009). This is a major drawback noted in the heat processing method. Hence, an alternative non-thermal treatment method that can modulate the structure of the native proteins, enhance the accessibility of the proteolytic enzymes to produce peptides with beneficial biological properties and can reduce the damage of phytochemicals would be more effective. At the same time, this alternative strategy can maintain the stability of the phenolic compounds. A widely used non-thermal processing technique that has been of high interest for researchers and is being studied as a potential alternative to heat treatments is High Pressure processing (HPP). HPP is popularly known to be used as a pasteurizing technique to inactivate microbes in food but it can also induce conformational changes and affect functionalities of food proteins (Cadesky et al., 2017). HPP has been reported to cause changes in the secondary and tertiary structures of different food proteins by the process of denaturation (Ye et al., 2017; Cadesky et al., 2017). Moreover, HPP treatment can also induce protein unfolding and thus can potentially enhance accessibility of the proteolytic enzymes to produce peptides (Zhang et al., 2012). The knowledge obtained from the existing scientific literature establishes the scientific premise of the present study and indicates that HPP pre-treatment could be an effective alternative strategy to produce bean-derived functional foods with enhanced antioxidant activity. It is

anticipated that HPP and proteolytic hydrolysis will be helpful to modulate the release of peptides with antioxidant activity as well as to retain the stability of the phytochemicals naturally present in the beans.

Dry edible beans from Nebraska is one of the best in the country and ranked in the top tier in the production of various beans like Great Northern beans (GNB), pinto beans, and red kidney beans. Among all the states of the United States, Nebraska leads the table in GNB production. GNBs are a type of dry edible beans that are typically kidney-shaped, medium-sized white beans and they are a good source of dietary proteins and phytochemicals. Therefore, GNB is a suitable candidate for the present project.

Parameters such as pressure and time are critical for HPP treatment. Pressure treatment at 600 MPa has been reported to induce structural alterations in proteins in soybeans (Tang and Ma, 2009). Duration of pressure applied is critical because excessive pressure for longer time induce protein aggregation and reduce the enzyme accessibility to digest proteins (Zhang et al., 2012). Gathering information from previous literature, we selected 600 MPa for 5 mins could be optimum to release peptides (Tang and Ma, 2009). Among a wide range of proteases that can generate peptides from food proteins, alcalase has been reported to be effective in cleaving proteins of the legumes like in mung beans (Li et al., 2005) or chickpeas (Li et al., 2008). Alcalase can generate peptides exhibiting biological properties.

Alcalase is an alkaline proteinase that functions at alkaline pH and is isolated from *Bacillus licheniformis*. Alcalase is known to have a wide range specificity while cleaving food proteins and can extensively release peptides with hydrophobic amino acid residues like phenylalanine(Phe), Tryptophan (Trp), Tyrosine(Tyr), Valine (Val), Isoleucine (Ile),

Leucine (Leu) or Methionine (Met) at the carboxyl terminal of the peptide sequences (Markland and Smith 1971). Branched chain amino acids at amino terminal and aromatic amino acid residues at carboxyl terminal of dipeptides and tripeptides can contribute towards enhance biological activity of peptides from food proteins (Cheung et al., 1980). Based on the above knowledge, 600 MPa pressure application was selected to be exerted for a duration of 5 mins for HPP treatment and alcalase as a suitable enzyme to generate peptides, while testing the proposed hypothesis.

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CHAPTER 2

LITERATURE REVIEW

2.1. INTRODUCTION

Functional foods are rich in essential nutrients like carbohydrates, proteins, fats, vitamins, minerals, and dietary fibers. These foods generally exhibit health-promoting properties, above and beyond their known nutritional value, that can help prevent several non-communicable chronic diseases (NCCD) like diabetes mellitus, obesity, coronary heart diseases, and cancers. The primary reasons for the increased rate of mortality and morbidity in the human population are mainly due to hypertension, diabetes, and other associated cardiovascular diseases (Chakrabarti et al., 2014, Bonow et al., 2002). The onset and progression of most of these chronic diseases are associated with enhanced inflammatory responses and an increase in oxidative stress. Unfortunately, limited options for pharmaceutical interventions are available to tackle the issues related to chronic increased of oxidative stress. Moreover, pharmaceutical interventions are often associated with adverse side effects, and that has triggered an increasing demand for some natural alternatives. Henceforth, researchers have been involved in identifying natural alternatives derived from plant or animal sources for similar therapeutic or preventative applications. Food substances that have health benefits above and beyond their known nutritional value that can prevent the progression of chronic diseases are now a great interest for future research. These active food-derived compounds are also known as ‘nutraceuticals’ (Keservani et al., 2015). The natural food sources that have high nutritional values and are a rich source of health-promoting bioactive compounds are listed as functional foods. Major examples of functional foods are milk, pulses, eggs,

meats, fishes, nuts, oats. The functional foods are characteristically rich sources for bioactive peptides, phenolic compounds, vitamins, and minerals that contribute to several biological properties like antioxidant, anti-inflammatory, anti-hypertensive, anti-cancer, and anti-microbial (Luna-Vital et al., 2015). Among the functional foods, pulses are a promising source of dietary proteins. It is an affordable health benefiting food source with high nutritional values. Pulses refer to the dry edible grains or seeds of legumes (FAO, 2016). Apart from being a high protein source, pulses have low sodium, low-fat content, but rich in iron, folate, and dietary fibers (Song et al., 2016). Pulses have a low glycemic index, which also proves it to be more health-promoting (Khandelwal et al., 2010). It has been previously reported that consumption of common beans has ameliorated the risks of obesity, cardiovascular diseases, colorectal cancer and, type II diabetes (Lanza et al., 2006; Campos-Vega et al., 2013; Câmara et al., 2013). As the most widely cultivated pulse crop in the state of Nebraska is the Great Northern Beans (GNB), this review intends to gather information about considering GNB as a source of bioactive compounds that could be health-enhancing. To archive the overall information, this review is divided into two sections that collects the up to date information and provide a critical analysis about dry edible beans as a functional food, effects of processing techniques and the release of bioactive peptides after enzymatic hydrolysis from beans, and the role of bean derived bioactive compounds in modulating the antioxidant activity. To understand the purpose of antioxidant activity of beans, we focus on oxidative stress, Reactive oxygen species (ROS) production during diseases, and the need for antioxidant defense by dietary sources like peptides in ameliorating several chronic diseases. Thus,

dry edible beans being rich in dietary protein could exhibit dietary antioxidant defense in ameliorating the harmful effect of excessive oxidative stress during disease pathogenesis.

2.2 Dry edible beans: Source of bioactive peptides

Dry edible beans have a total protein content ranging from 16-33% and are a very cost-effective, food protein source to derive bioactive peptides (FAO, 2016). These beans have several storage proteins that can be cleaved by several methods, to derive bioactive peptides. Beans majorly have two types of proteins: the albumins and globulins. Albumins are the water-soluble metabolic proteins, compared to globulins that contain higher concentrations of the essential amino acids like tryptophan (Trp), lysine (Lys), threonine (Thr), and methionine (Met). Globulins are considered to be salt-soluble storage proteins and can be further subdivided into legumin and vicilin proteins, with minor amounts of a third type known as convicilin (Carbonaro, 2006). The major globulin type of protein found in beans is phaseolin (50 kDa) which is also the most abundantly found storage protein in beans. Besides phaseolin, the other proteins found in beans are lectins (33 kDa), phytohemagglutinins (25 kDa), and acrelins (29 kDa) (Garcia-Mora et al. 2016, Janarthanan et al. 2012). These proteins can be isolated from the beans by process of extraction or can be enzymatically cleaved to derived bioactive compounds(Satterlee et al., 1975). Besides being rich in proteins, beans are also rich in phytochemicals and this contributes towards the biological properties of beans (Oomah et al., 2005). Presence of phytochemicals have been shown to enhance the antioxidant properties of food proteins (Tang & Tsao, 2017). Thus, people across the world, prefer to include beans as a part of their daily diet due to these above discussed important

components present in beans. The most preferable way of bean consumption is by boiling the beans. Often soaking precedes boiling to make the beans softer and also to improve the quality of beans by removal of certain anti-nutritional factors present in the beans (Huma et al., 2008). Soaking and boiling are primary processing methods that can facilitate the digestion of bean proteins and can affect the stability of phytochemicals present in the beans (Mecha et al., 2019).

2.2.1 Cultivation and Processing of Great Northern Beans (GNB)

The United States is listed as the global leader in the production of dry edible beans in the world. Nebraska ranks 3rd among the states after North Dakota and Michigan in the production of beans. About five types of beans are extensively cultivated in the USA and consumed by the human population at large. These include the Pinto beans, Black beans, Chickpeas, Red kidney beans, Great Northern beans, and Navy beans. Great Northern Beans are one of the most extensively grown dry edible beans in the state of Nebraska and listed among the top producers of Great Northern Beans (GNB) in the United States. Nebraska contributes towards 85% of the total production of GNB, nation wise. The main regions that rank highest in the production of dry edible beans in the United States are the central high plains that span across Colorado, Wyoming, and Nebraska. Production of GNB is extensive in the Western part of Nebraska (popularly known as the bean belt), mainly in in Panhandle regions as the semi-arid weather conditions are favorable for bean production.

Processing of beans is a critical factor that must be considered for beans to be a reliable food protein to derive bioactive peptides. The boiling of beans is preferred as a strategy to

consume the beans as it improves the digestibility of the beans. However, the thermal processing of food proteins affect the protein quality, release of bioactive peptides as well as their antioxidant capacity (Remanan & Wu, 2014). The cooking of beans has been reported to have inactivated some of the protease inhibitors and lectins present in beans (Luna-Vital et al., 2015). Besides, heat treatments, non-thermal treatment such as High Pressure processing (HPP) are also being studied as a promising alternative. HPP pre-treatment or the HPP assisted enzymatic hydrolysis needs further research to understand its efficiency in the production of bioactive peptides as it can possibly enhance hydrolytic yields and aid in the reduction of the reaction time (Garcia-Mora et al. 2016). However, the effect of high pressure treatment on the aspect of a generation of peptides and their biological activity has not been studied yet. This review focuses on the effects of thermal and non-thermal processing has been studied so far in terms of processed bean derived bioactive compounds such as bioactive peptides.

2.3 *Food Derived Bioactive Peptides*

Proteins present in functional foods are source to derive biologically active compounds called bioactive peptides that are in an inert form within the native protein but can exert health-enhancing properties upon the release from the native protein (Rizzello et al., 2017). Research related to the food-derived bioactive peptides has rapidly advanced in the last few decades. These peptides are popularly known to be health-promoting due to its high tissue specificity. Natural sources of bioactive peptides can be from animals, plants or microbes as these organisms have proteins. The traditional process used to derive bioactive peptides from natural food proteins is hydrolysis by proteases that can

specifically cleave the food proteins to generate bioactive peptides with biological properties (Abdel-Hamid et al., 2017). Bioactive peptides derived from food proteins by the use of proteases have been shown to exhibit health-promoting biologically activities like anti-inflammatory activity, anti-proliferative activity, antioxidant-activity, anti-hypertensive activity (Udenigwe & Aluko, 2012). Enzymes that are chosen in a way so that it can specifically cleave certain storage proteins in order to expose key amino acids like aromatic or basic residues that may contribute to the biological activity of the peptides (Zheng et al., 2016). Peptides can exhibit a wide range of biological properties and for this reason, they are being studied as a probable therapeutic intervention to treat several chronic diseases (Hartmann & Meisel, 2007). Hence, research suggests that the typical sequence of the peptides produced from a native food protein primarily depend on the enzymes used for proteolysis and their cleavage mechanisms (Mojica et al., 2018). Literature studies revealed that lower molecular weight bioactive peptides can exhibit better antioxidant activity than the higher molecular weight peptides (García-Tejedor et al., 2014). The bioactive peptides can be derived by enzymatic hydrolysis with enzymes like papain, alcalase, thermolysin or more popularly by the gastrointestinal (GI) enzymes like α-amylase, pepsin, pancreatic lipase, trypsin and chymotrypsin (Li et al., 2005, Singh et al., 2017). For the process of enzymatic hydrolysis, enzyme extracts used are either derived from animal sources (e.g., trypsin, pepsin) or microbes (e.g., Alcalase®, Neutrase®). Some plant proteases are also used like papain and bromelain. Enzymes used from bacterial sources like alcalase from *Bacillus licheniformis* is a subtilisin enzyme that acts at an alkaline pH. Subtilisins belong to the group of serine proteases that can cleave a peptide bond by nucleophilic attack by the serine residue at the active site. The molecular

weight of subtilisins range from 20-45 kDa. Alcalase has been reported to cleave proteins non-specifically and expose essential hydrophobic as well as aromatic amino acids in the peptide sequences (Sarmadi & Ismail, 2010) which have been essential for the biological properties exhibited by peptides. Alcalase cleaves food proteins in a catalytic triad mechanism that involves a charge relay network of Asp-32, His-64, and the active site Ser-221. The Ser-221 residue acts as a nucleophile and cleaves peptide bonds with its partially negative oxygen atom. This is possible due to the nature of the charge-relay site of subtilisin (Siah et al., 2014). The measure of the total peptide bonds cleaved during enzymatic hydrolysis is the Degree of hydrolysis or DH (Adler-Nissen, 1986). The degree of hydrolysis (DH) of proteases can be measured by different methods such as pH-Stat method, TNBS (Trinitrobenzene-sulfonic acid), or OPA (*o*-phthaldialdehyde) methods. Measurement of DH after alcalase hydrolysis was measured and it has been reported that the DH value calculated by pH-stat and TNBS method was higher than that calculated by the OPA method (Spellman et al., 2003). However, there are factors that could be affecting the release of bioactive peptides or simply, the DH of the beans could be affected by several factors like bean processing (Huma et al., 2008; Evangelho et al., 2016) or growth conditions of the beans, related to the overall purpose of the study.

2.4 *Effects of Different Processing Methods*

2.4.1 *Effects of Boiling of beans*

Boiling beans can denature the storage proteins present in beans and increase the exposure of the cleavage sites for the accessibility of the proteases in digesting the proteins to generate bioactive peptides (Xu & Chang, 2009). Boiling, or cooking, time is

important in terms of consumption and absorption of beans retaining all the nutritional values. Denaturation of the storage proteins like phaseolin takes place post thermal treatment, thereby exposing the hydrophobic amino acid residues of the native bean proteins on enzymatic hydrolysis (Montaya et al.,2008). Previous studies show considerable there is a remarkable increment of total phenolic content and antioxidant activity after thermal processing of several kinds of bean varieties (Oomah et al., 2005). However, heat treatments have also been reported to have reduced the amount of phytochemical present (Ketharin et al., 2019). Although beans naturally possess immense nutritional value, it requires considerable cooking time to be palatable for consumption (Cichy et al., 2015). Storage of beans can also impact the extent of cooking time. Soaking beans helps in better and faster cooking compared to directly cooking dry edible beans (Arruda et al., 2012). Dry beans essentially consist of certain components called anti-nutritional factors that include phytates, saponins, lectins, tannins (Doria et al., 2012). After the soaking process, the draining out of soaking water helps to improve the quality of the beans by removing some of the anti-nutritional factors present in beans (Zamindar et al., 2013). This could also be beneficial for improving the enzymatic hydrolysis of food proteins by removing some of the trypsin inhibitors present in beans (Huma et al., 2008). Beans that are cooked immediately after harvest may cook faster as compared with beans stored for six months to 1 year (Galdino Alves et al., 2016). Beans stored at high temperatures tend to require more time to be properly cooked and referred to as ‘hard-to-cook' beans (Tuan & Phillips, 1992). Previous literature studies reveal that longer cooking due to ‘hard to cook’ properties of beans may reduce the nutritional value and protein quality of the dry edible beans (Tuan & Phillips,1992). Considering some of

the drawbacks of heat processing, like the destruction of phytochemicals, we went to gather information on an alternative to heat treatment that is High Pressure processing or HPP.

2.4.2 *Effects of High Pressure Processing (HPP)*

Functional properties of a food proteins can be affected due to various preparatory stages, processing conditions such as a high temperature or application of high pressure, storage, and also due to ways of consumption (Messens et al., 1997). Pressure treatment on food proteins can induce conformational changes such as protein denaturation or tendency to aggregate via hydrophobic interactions (Tang & Ma, 2009). Other factors that can influence the function of a food protein are the duration of high-pressure treatment and the intensity of pressure applied (Messens et al., 1997). Application of high pressure causes the internal cavities to get compressed, thus decreasing the cubic measure of the protein of interest. Previous literature has stated that high pressure processing induced aggregation leads to enhanced functionalities like emulsifying property of lupin proteins (Chapleau & De Lamballerie-Anton, 2003). The study by Tang and Ma (2009) shows that there was soluble as well as insoluble protein aggregation after the High Pressure processing at 200-600 MPa. However, at the pressure of 600 MPa, the formation of soluble aggregates was by far more than the insoluble aggregate. Tang and Ma (2009) proved, by carrying out an FTIR (Fourier transform infrared spectroscopy) that depicted moderate unfolding of native proteins according to the level of pressure applied and a reversion of the process of unfolding was observed on the pressure being released (Tang & Ma, 2009). HPP treatment can enhance accessibility of digestive enzymes to cleave

food proteins similar to the process of boiling (Chao et al., 2013). On the application of high pressure, aggregation takes place due to the formation of disulfide bonds in the food protein (Messens et al., 1997). It has been reported that different processing methods could have a positive impact on the release of bioactive peptides from food proteins as it removes the anti-nutritional factors and can aid in protein denaturation (Bentacur-Ancona et al., 2014). It has also been reported that processing methods could affect the biological activities exhibited by the food protein derived bioactive peptides (Chao et al., 2013). In this review, we further tend to focus on antioxidant activity of the bioactive compounds. The major consequences that follow along with the pathogenesis or progression of some diseases is the surge in the concentration of reactive oxygen species (ROS) in the infected cellular environment. Surplus levels of ROS in cells increase the oxidative stress that could otherwise be harmful to the health and metabolism of the cells. This particular medical condition demands an external source to exhibit antioxidant defense (Cam & de Mejia, 2012).

2.5 *Oxidative Stress and Reactive Oxygen Species*

Oxidative stress is the condition that takes place if there is an imbalance in the amount of free radicals present in the cellular environment, due to the disability to scavenge or detoxify naturally by the cells (Ahmadinejad et al., 2017). This stressed environment could be harmful to the proteins, lipids, and DNA (Chandra et al., 2015). Oxidative stress could be induced by exogenous or endogenous sources. The sources that are considered to be exogenous are ionic radiations, pesticides, or any environmental chemicals. Oxidative stress that is induced by metabolism, signaling pathways or by inflammatory

response is referred to as endogenous sources to the cells (De Bont & van Larebeke, 2004). The form of oxygen that is produced in a biological system and has both destructive and, a beneficial role in the system are the ROS (Figure 1.1). ROS is produced by a sequence of enzymes like NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, xanthine oxidase, eNOS (endothelial nitric oxide synthase) lipoxygenase, enzymes of Cytochrome P450 and also by respiratory chain in mitochondria (Ahmadinejad et al., 2017). Reactive oxygen species at low levels play a significant role in the regulation of several cellular signaling pathways (Marosi et al., 2012). Inflammation induces the efflux of reactive oxygen species which induces oxidative stress in the cellular environment (Zuo et al., 2019). During the pathogenesis of the inflammation-associated diseases, ROS performs a protective role, diminishing the harmful effect due to the increase in oxidative stress in the cellular environment. The onset of chronic diseases like cardiovascular diseases, diabetes mellitus, Alzheimer's disease and forms of cancer are marked with oxidative damage to the cellular system that take place due to increased accumulation of ROS (Zuo et al., 2019). For example, ROS plays a vital role in restoring the endothelium homeostasis between vasoconstriction and vasodilation associated with cardiovascular diseases (Montezano et al., 2015). Similarly, in the progression of some of the age-related diseases, an increase in oxidative stress leads to the activation of several pro-inflammatory pathways related to the progression of the diseases (Reuter et al., 2010). As a defense mechanism during pathogenesis of a disease, inflammation activates phagocytes to produce free radicals that can fight against the microorganism that has caused the infection (Chen et al., 2012). The rise in ROS levels can be correlated to the oxidation of low-density lipoprotein (LDL), symptoms

present in cardiovascular diseases (CVDs) (Cam & de Mejia, 2012), and endothelial dysfunction (Keaney et al. 2003 , Rao et al. 2010). It is known that ROS includes hydrogen peroxide, superoxide, singlet oxygen and hydroxyl radicals that can also initiate and regulate the cell death process termed as autophagy (Chen et al., 2009). Among the types of ROS, superoxide is produced as a consequence of immune response against pathogenesis by microorganisms. Superoxide anions can produce other active free radicals that on interaction with macromolecules in the biological system initiate tissue damage (Halliwell & Gutteridget, 1984). Some of the flavonoids exhibit antioxidant activity by scavenging superoxide radical anion. These ROS are produced by a series of chemical reactions that are accelerated by cellular enzymes identified in the biological system such as superoxide dismutases (SODs) and glutathione peroxidase (GPX). Catalases found in peroxisomes act on hydrogen peroxide and converts it to water and oxygen (Chelikani et al., 2004). The surge in ROS is due to diminished natural antioxidant defense in the cellular environment. Besides these enzymatic antioxidant components, dietary sources like proteins, peptides, carotenoids, flavonoids, polyphenols, and vitamins found in functional foods are listed as non-enzymatic antioxidants that can maintain the required levels of ROS in the biological system (Liu et al., 2018). Figure 2.1 is a simplified illustration of the process by which oxidative stress increases and is reduced by ROS scavenging by antioxidant defenses. However, the antioxidant activity of these non-enzymatic components especially bioactive peptides could be affected by several factors such as processing methods, or the hydrolytic enzymes used to release the peptides from proteins or the structural composition of the native food proteins that could be affected by growing conditions.

2.6 *Antioxidant Activity of Bioactive Compounds*

The sources that are categorized as antioxidant defenses in the biological systems are superoxide dismutase, glutathione peroxidase, albumin, ferritin, ascorbic acid, tocopherol, carotenoids, phenols or hormones like estrogen, melatonin, and angiotensin. Thus, food sources rich in these components can prevent diseases because of their antioxidant properties (Wu et al., 2004). Processing of beans can affect the release of the peptides from native protein sources and their antioxidant activity. Cooking beans was reported by Wu et al. (2008) to have diminished the antioxidant activity, which could be a result of the denaturation of proteins (Wu et al., 2008). Besides processing, the antioxidant activity of bioactive peptides is affected by the type of protease used to cleave the proteins and the degree of hydrolysis (Penta-Ramos & Xiong, 2002).

The functional R groups such as indole or imidazole or phenol groups present on the aromatic amino acid residues (Trp, Tyr, Phe) or the basic residues (Arg, His) may contribute to different biological activity exhibited by peptides released by enzymatic hydrolysis (Sarmadi & Ismail, 2010) from food proteins. Peptide sequences that have aromatic amino acid residues or basic residues, behave as proton donors to scavenge free radicals and maintain the molecular stability (Sarmadi & Ismail, 2010). Amino acids Leucine, Valine , Alanine or Isoleucine on the N-terminal of the produced bioactive peptides significantly contributes in the antioxidant activity of peptides (Li et al. 2011, Guo et al.,2009) . Literature also suggests that the most of the antioxidant peptides obtained from bean hydrolysates, are typically small in size (< 1 kDa) and may consist more of the afore-mentioned amino acids at the N- and C-terminals (Luna-Vital et al., 2015). The other factor that is essential for the antioxidant activity of beans is the

phenolic composition of the beans. Besides being a prominent source for proteins, beans are found to be rich in other components that aid in the high bioactive properties of beans. Beans have been shown to have phenolic compounds, flavonoids, and also anthocyanins that may be beneficial in reducing oxidative stress during infection (Díaz-Batalla et al., 2006). The components in plants that have one or more phenol units along with hydroxyl substituents are a kind of secondary products present in plants, widely known as phenolics (Akyol et al., 2016). Phenolic compounds that one hydroxyl group along with one or more aromatic ring are called the phenolic acids such as ferulic acid, caffeic acid, cinnamic acid, p-coumaric acid, protocatechuic acid. However, the most abundant polyphenols found in beans, consist of at least two or more aromatic rings and one or more hydroxyl groups that are attached to a heterocyclic pyran group and these are the flavonoids. Phenolic compounds can scavenge free radicals or chelate redox metals and can also help in the signaling of cellular responses (Pham-Huy et al., 2008). Ferulic acid is the most important phenolic compound that has been shown to have antioxidant activity as well due to high free radical scavenging, singlet oxygen quenching capacity, transition metal chelating properties (Craft et al., 2012). The diverse colors observed in the common beans may be due to the presence of phenolics (Nayak et al., 2015) The risk of diabetes can notably be ameliorated by anthocyanins (Wedick et al., 2012). Processing or enzymatic hydrolysis of the bean proteins can affect the phenolic content of the beans and henceforth affect the antioxidant properties of the bean derived peptides (Oomah et al., 2005). Phenolic composition in beans could vary due to processing like soaking, cooking, or pressure application (Siah et al., 2014). Leaching of some phenolic compounds may take place during the process of soaking or during the boiling of beans

(Siah et al., 2014). The antioxidant activity of bioactive compounds such as peptides are popularly measured by spectrophotometric methods of ABTS radical scavenging assay and Oxygen Radical absorbance capacity (ORAC) (Gülçin et al., 2010).

2.6.1 *Measurement of Antioxidant Activity of Peptides*

Antioxidant activity of the bean derived bioactive peptides could be described as the ability of the bean protein hydrolysates produced by enzymatic hydrolysis to scavenge the free radicals present in the cellular environment. Several methods are used to determine the antioxidant capacity of bioactive compounds derived from food proteins. Antioxidant capacity is evaluated either by measuring the hydrogen atom transfer (HAT) method or by the electron transfer (ET) method (Huang et al., 2005). In the first method HAT, the antioxidant compounds compete with a substrate to reduce the generation of free radicals. In this process, decomposition of azo compounds takes place. The antioxidant capacity is measured by the change in color as a result of the reduction of an antioxidant. The intensity of the color is proportional to the concentration of the antioxidant present in the test sample (Zulueta et al., 2009). A bioactive compound can inhibit the release of reactive species like ROS or scavenge them. A potent antioxidant can also enhance the endogenous defense mechanism by primarily influencing the expression of genes that code for the antioxidant sources present in the biological systems (Cadenas and Packer, 2002). Radical scavengers can inhibit a peroxidation reaction by quenching the peroxide radicals. [2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) radical scavenging assay is the electron transfer method to detect the antioxidant activity of compounds. A stable blue-green ABTS chromophore is produced in a direct

reaction of ABTS with potassium persulfate and is scavenged to produce a colorless compound. The intensity of decolorization is used to measure the concentration of radical scavenged to determine the antioxidant activity of the sample (Re et al., 1999). The oxygen radical absorbance capacity (ORAC) method used to measure the antioxidant activity of peptides derived from food proteins is an established and accurate test. In the ORAC antioxidant assay, oxidation induced by peroxyl radical is inhibited by the decomposition of AAPH (2,2'-azobis (2-methylpropionamide)-dihydrochloride) which is also a kind of azo compound. This method is implemented to determine the antioxidant activity of food proteins extracts or food-derived bioactive peptides.

2.7 CONCLUSION

From the background information gathered so far and considering the key concepts behind the thermal and non-thermal processing methods, food derived bioactive peptides and their role as the dietary antioxidant defenses, the experimental design to achieve the hypothesis of this study was designed. As learnt from the literature, heat treatments as well as pressure treatments could enhance the digestibility of beans. Literature also showed that heat treatment could have a destructive effect on the phytochemical content naturally found in beans. From this information, HPP was chosen as the possible alternative to heating that could also be able to prevent the degradation or loss of phytochemicals from the beans. Based on the scientific knowledge obtained, we predict that both high pressure treatment and enzymatic hydrolysis can produce peptides as well as enhance the stability of phytochemicals and improve the antioxidant activity of the beans. Based on the review study, we design our experimental flow and parameters to

achieve the above stated hypothesis. We select 600 MPa for 5 mins for the HPP treatments and alcalase as the protease to generate peptides with potent biological activity.

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FIGURES

Figure 2.1

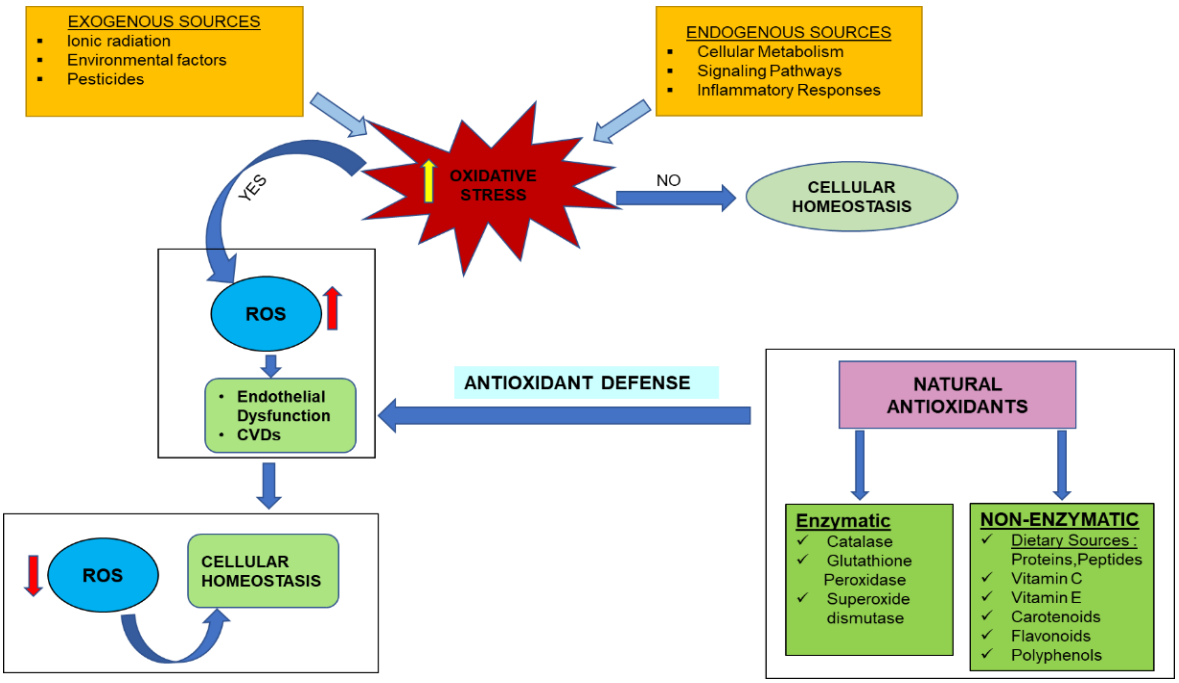

Figure 2.1: A schematic representation of oxidative stress, ROS and Antioxidant Defense.

CHAPTER 3. EVALUATING THE EFFECT OF HIGH PRESSURE PROCESSINGIN CONTRAST TO BOILING ON THE ANTIOXIDANT ACTIVITY FROM ALCALASE HYDROLYSATE OF GREAT NORTHERN BEANS (*P. vulgaris)*

3.1 INTRODUCTION

Common beans (*Phaseolus vulgaris*), a part of the leguminous family (*Fabaceae*), are the most widely grown and consumed pulse globally, particularly in South America, Asia, and Europe. Besides being economical, beans are an excellent source of proteins, carbohydrates, dietary fibers, micronutrients, and phytochemicals, and thus are still an irreplaceable source of nutrition for millions of people around the world. Dry edible seeds are referred to as pulses and in the United States, pulses are gaining popularity due to the consumers' growing interest in plant-based proteins, and common beans are one of the best sources of plant-based proteins. Moreover, if population growth assumptions are correct, protein needs will significantly increase from 32 to 43% in the next three decades (Henchion et al., 2017). Thus, the interest in plant-based proteins is also expanding. In addition to this growing demand, consumption of common beans is also highly sought after because of the health beneficial effects attributed by its nutritional components and bioactive compounds. Peptides derived from common bean proteins are bioactive, rendering antioxidant, anti-hypertensive, anti-inflammatory, and anti-cancer activities (Chen et al., 2019). Furthermore, the major phenolic compounds found in common beans, such as phenolic acids, flavonols, flavan-3-ols, anthocyanidins, and flavanones, are also considered bioactive and have been shown to improve health *via* an array of actions such

as antioxidant, anti-hypertensive, anti-inflammatory, and anti-atherosclerotic activities (Del Rio et al., 2013). The combination of such compelling bioactive profiles and its ever-increasing evidence of health beneficial effects have led researchers to seek new techniques to produce more stable and bio-accessible bioactive compounds from beans. Since beans are an excellent source of low-cost protein source, it is also a potent source of producing peptides with health beneficial biological activities after enzymatic hydrolysis. Several factors influence the generation of peptides from common beans, including bean variety, hydrolysis conditions, pre-treatments, protein extraction, and enzyme specificity (Luna-Vital et al., 2015). In terms of enzyme specificity, alcalase enzyme, an endo-protease, has been widely used for the generation of bioactive peptides due to its broad specificity on the proteins and, thus, greater chances to generate a wide diversity of peptides (Torruco-Uco et al., 2009). However, a major challenge which emerges during the preparation of protein hydrolysates from common beans is the high resistivity of phaseolin (prime seed storage protein) towards enzyme hydrolysis (Rui et al., 2012) and the phenolic-protein interactions, which also decrease the hydrolytic efficiency (Flurkey et al., 2008; Tan et al., 2011). Therefore, to enhance the hydrolytic efficiency of beans, several strategies and pre-treatments have been investigated (Rui et al., 2012). Common beans require full hydration or soaking before any treatment process to reduce the tannin (anti-nutritional compound) content and reduce the boiling time (Belmiro et al., 2020). The most common pre-treatment applied to dry beans since humankind started considering them as food is applying high heat (boiling temperature) to cook the beans. Boiling changes the beans' physical characteristics and chemical composition enhances susceptibility of proteolytic digestion, but it also results in the

reduction or damage of the phenolic compounds. On the other hand, High Pressure processing(HPP) is an emerging and alternate non-thermal processing treatment that has been successfully used to enhance proteolytic digestion and generate bioactive peptides from chickpea, lentil, and pea (Chao et al. 2013, Li et al. 2008). The combination of HPP and enzymatic hydrolysis is worth examining for the production of bioactive peptides as it can enhance hydrolytic yields and reaction time (Garcia-Mora et al., 2016). Since limited information is available about the effect of HPP treatment and enzymatic hydrolysis to the release of bioactive peptides and also in maintaining the stability of the phenolic compounds in beans, this work is mainly focused on comparing these two pre-treatment methods for the generation of peptides with potential antioxidant activity from alcalase digested Great Northern bean (GNB) hydrolysates and to evaluate the efficacy of the HPP pre-treatment to maintain the stability of phytochemicals present in beans after enzymatic hydrolysis.

3.2 MATERIALS AND METHODS

3.2.1 Great Northern Beans (GNB)

GNBs are grown in the Panhandle region (Panhandle Research Status) of Nebraska were obtained from the laboratory of Dr. Carlos Urrea at the University of Nebraska-Lincoln. The dry beans were stored at room temperature after harvesting and used within six months for testing.

3.2.2 Processing of GNB

a. Control Treatment (Trt-1): Soaking and boiling of the beans

Beans were soaked in double-distilled water (1:2 ratio) for 12-16 h, overnight at 4 °C. After that, the beans were drained, placed in double-distilled water (ratio 1:2), and cooked at 90 °C for 80 min. The temperature was monitored continuously using a thermometer. After boiling, the water was drained, and the cooked beans were cooled down to room temperature and eventually stored at -20 °C until further processing.

b. Treatment 2 (Trt-2): Soaking and High Pressure processing (HPP) of beans

Beans were soaked similarly, as mentioned in Section 2.2a. After straining the soaking water, the soaked beans were placed in vacuum-sealed pouches before being subjected to pressure treatment. An isostatic pressure of 600 MPa for 5 min at room temperature was applied using a Hiperbaric 55 L system (Hiperbaric USA, Miami, FL). Afterward, the beans were stored at -20 °C until further testing. The high pressure processing was replicated twice.

c. Treatment 3 (Trt-3): Soaking, HPP treatment, and boiling of beans

Beans were soaked and HPP-treated, as mentioned in Sections 2.2a and 2.2b. Afterwards, the high pressure-treated beans were cooked at 90 ^{o}C for 80 min and stored at -20 °C until further testing.

3.2.3 Extraction of proteins from processed beans

The frozen processed beans (Trt-1 x 2, Trt-2 x 2, Trt-3 x 2) were weighed up to 5 g and thawed at room temperature for 1-2 h. After thawing, the beans were ground using a mortar and pestle and suspended in a 10% (w/v) solution using 2% (w/w) sodium

chloride (NaCl) solution. Protein extraction proceeded using the same methods used by Satterlee et al. (1975). For 5 g of beans, 50 mL of NaCl solution was added. Thereafter, the suspension was stirred at room temperature, overnight (12-16 h). The following day, the samples were centrifuged at 10,000×g for 40 min to obtain the supernatant consisting of the isolated proteins, while the pellet was discarded. The supernatant was lyophilized in a freeze-drier (Catalogue No. 794801000, Labconco, Kansas City, MO) and stored at -20 °C until further analysis.

3.2.4 Enzymatic hydrolysis of processed beans

Following processing about 100 g of each of the processed beans were minced using a kitchen mincer for alcalase digestions. For the alcalase digestion, 2.5 % of protein slurry was prepared (approximately 24 g of beans was weighed; based on protein % present in beans, and suspended in 200 mL of double-distilled water). The slurry was vortexed well and heated at 85 °C for 10 min to inactivate any endogenous enzymes. Afterwards, the slurry was placed in a circulating water bath at 60 °C (using a circulating water bath). Using a Titrando 902 pH-STAT coupled with an 800 Dosino device (Metrohm AG, Herisau, Switzerland), pH was maintained at 8.5. After 5 min of equilibration, 0.2 mL alcalase (#126741, Millipore Sigma, Burlington, MA) for every 5 g protein was added to the protein slurry to yield 4% (v/w) of enzyme per protein concentration. The slurry was allowed to hydrolyze for 3 h following which the activity of the enzyme was inhibited by lowering the pH from 8.5 to 4 using 1 M hydrochloric acid (HCl). The hydrolysate was collected and centrifuged at 10,000×g for 40 min. The supernatant was collected for freeze-drying and then frozen at -20 °C until further testing. The alcalase digestion of

each of the processed beans was done in duplicates, thus referring these as the subsamples of processed beans after digestion. The pellet was discarded, and the supernatant was stored to further analysis.

The degree of hydrolysis (DH) was determined by the pH-STAT method calculation based on the following equation (Mat et al., 2018):

$$DH(\%) = \frac{V_{NaOH} M_{NaOH}}{M_p h_{tot}} \cdot \frac{1}{\alpha_{NH_2}} \cdot 100\%$$

where V_{NaOH} is the titrant volume (mL), M_{NaOH} is the molarity of the titrant (M), M_p is the protein mass (g), h_{tot} is the number of peptide bonds per gram of protein (7.8 for beans) (Bamdad et al., 2009), and α_{NH_2} is the mean degree of dissociation for the amino groups (0.44) (Mat et al.,2018). The titrant used in this alcalase enzymatic hydrolysis was 1 M NaOH.

3.2.5 Estimation of the total protein content

Protein estimation was carried out following the conventional protocol as proposed by Lowry et al. (1951). The reagents used were 1% copper sulfate solution (Reagent A), 2% sodium tartrate solution (Reagent B), and 2% sodium carbonate solution (Reagent C). Aliquots of 0.5 mL each of Reagent A and Reagent B were added to 100 mL of freshly prepared Reagent C and the mixture was referred to as Reagent D. The Folin's reagent was diluted with water in the ratio 1:1. The stock solutions (1 mg/mL) of the freeze-dried processed protein isolates were prepared using double distilled water. For the assay, 100 µL of water (blank sample), the stock solutions of the protein isolates, and protein standard solutions based on bovine serum albumin (BSA) were added individually to

tubes each containing 1 mL of Reagent D and was immediately mixed using a vortex. The mixtures were incubated at 37 °C for 10 min. Thereafter, 100 µL of the diluted Folin's reagent were added to each of the tubes, mixed, and incubated under dark conditions at 37 °C for another 30 min. Afterwards incubation, the absorbance at 660 nm (A_{660}) were measured using the microplate reader (Model Synergy H1, BioTek US, Winooski, VT) The protein content (µg/mg) of the processed bean protein isolates were conducted in triplicates in the well plate and assayed twice. Calculations were done using a standard curve generated from the A_{660} values of the Bovine serum albumin (BSA) standard solutions.

3.2.6 Estimation of peptide content

The freeze-dried hydrolysates were weighed to prepare 1 mg/mL stock solutions using double-distilled water. Samples were then prepared by diluting the stock solutions to 125 µg/mL. The peptide content (µg/mg) of each sample was measured in triplicates using the Pierce™ Quantitative Fluorometric Peptide Assay (Catalog No. 23290, Thermo Scientific, Waltham, MA) and a microplate reader (Model Synergy H1, BioTek US, Winooski, VT).

3.2.7 Antioxidant activity by ABTS radical scavenging assay

ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical scavenging assay was performed following the method proposed by Re et al. (1999) with some minor modifications. ABTS radical cation was produced by reducing 7 mM of ABTS stock solution with 2.45 mM potassium persulfate. The mixture solution was kept in dark at room temperature for 16 h before use. ABTS solution was diluted with 100 mM

phosphate buffer saline (PBS) to an absorbance of 0.7 ± 0.02 A.U. at 734 nm using a microplate reader (Model Synergy H1, BioTek US, Winooski, VT).

Freeze-dried hydrolysates were suspended in 100 mM PBS solution to the following concentrations: 0.5, 0.25, 0.125, 0.062, and 0.031 mg/mL. Standard solutions of Trolox (Catalog No. 238813, Millipore Sigma, St. Louis, MO) were prepared in 100 mM PBS to obtain a standard curve and 100 mM PBS was used as the control or blank sample. For the assay, 20 µL each of control, Trolox standard (Catalog No. 238813, Millipore Sigma, St. Louis, MO), and hydrolysate sample solutions was added dispensed in wells of a 96-well plate, followed by 200 µL of the diluted ABTS solution. Absorbance at 734 nm (A_{734}) were recorded and a standard curve based on the A_{734} nm vs. Trolox concentration was plotted. The inhibition of the ABTS radical was calculated using following the formula:

$$ABTS^{\bullet+} \text{ scavenging effect } (\%) = \frac{A_c - A_s}{A_c} \times 100\%$$

Where, A_c is the absorbance at 734 nm of the Control (100 mM PBS) solution, A_s is the absorbance of a sample at 734 nm. Based on the $ABTS^{\bullet+}$ inhibition (%) data and Trolox standard curve, the IC_{50} values, which correspond to the peptide concentration for 50% inhibition of the ABTS radical formation, were obtained and used for further analysis.

3.2.8 Antioxidant activity by Oxygen radical absorbance capacity (ORAC)

The antioxidant activity of the bean-derived hydrolysates from the three processing treatments were measured following the method by Remanan & Wu (2014) along with some modifications to the original method by Ou et al. (2001). Freeze-dried hydrolysates were weighed to make 1 mg/mL stock solutions in 75 mM PBS, which were further

diluted to 62.5 µg/mL for the ORAC assay. Standard solutions of Trolox (Catalog No. 238813, Millipore Sigma, St. Louis, MO) in 75 mM PBS were used to generate a standard curve, with 75 mM PBS used as a blank. Hydrolysate samples were tested in duplicates. The antioxidant activity was represented by measuring the Trolox equivalent antioxidant capacity of each of the samples.

3.2.9 SDS-PAGE analysis

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was conducted using the freeze-dried bean-derived proteins and hydrolysates and a 4-20% gradient gel. The protein isolates of processed beans as well as the corresponding hydrolysates were weighed to make a stock solution of 12 mg/mL with double distilled water. This stock solution was further diluted to get a final concentration of 2 µg/µL after dilution with the 2X Laemmli sample buffer (Catalog No. 1610737, Bio-Rad, Hercules, CA) as a loading dye. The pre-digested, processed beans, as well as the processed whole bean alcalase digested hydrolysates were separated using a 4-20% gradient gel (Catalog No. 4561096, Bio-Rad, Hercules, CA). Precision plus protein standards dual-color protein ladder (Catalog No. 181-0374, Bio-Rad, Hercules, CA) was used as the protein marker and 0.1% Coomassie R250, along with 10% acetic acid and 40% methanol, were used to stain the gels. Later, the staining solution was replaced with a de-staining solution (20% methanol and 10% acetic acid) for 2 h. The gels were intermittently washed with water until the dye stain faded away. The gels were analyzed to identify the separate bands of the proteins present in the samples using an infrared imaging system, Odyssey$^{@}$ CLX Imaging System (Li-Cor Biosciences).

3.2.10 Characterization of peptide fractions

An aliquot of the freeze-dried hydrolysates was resuspended in water at a concentration of 20 µg/µL. All the samples were injected twice to have technical duplicates. These samples were serially diluted ten times with 90% acetonitrile and transferred into fresh vials. The smaller peptides (length: < 4amino acids) were separated by Hydrophilic Interaction Liquid Chromatography (HILIC) and the longer peptides (length: > 4amino acids) were separated by Reverse Phase (RP) Liquid Chromatography. For the HILIC separation of the peptides with 2-3 amino acids, the samples were further diluted twice for a 50µg injection. The separation of the peptides was done on X Bridge Amide 3.5 µm (4.6 x 100 mm, Waters, Milford, MA) column using a Vanquish (Thermo Fisher Scientific, Waltham, MA) HPLC at 45°C and at a flow rate of 400 µL/min with a gradient of A (0.1% formic acid in 100% LC-MS grade water) and B (0.1% formic acid in 100% acetonitrile) as follow: 90% B to 30% B in 13 min, then back to 90% in 0.5 min. The data was acquired on a QE-HF (Thermo Scientific, Waltham, MA) mass spectrometer using positive ion mode. The mass range used was 60 to 900 m/z on single charge ions at 60,000 resolution, using an AGC (automatic gain control) target of 3e6 and a maximum ion time of 50 ms. The isolated ions were further fragmented by HCD (high-Energy collisional dissociation) using isolation window of 1.6 m/z at a resolution of 15,000. For the separation of the larger peptides, samples were diluted to 2 times and run by reverse phase (RP) on a ACCQ-TAG ULTRA C18 1.7 um (2.1 x 100 mm, Waters, Milford, MA) column using a Vanquish (Thermo Fisher Scientific, Waltham, MA) HPLC at 40°C and at a flow rate of 300 µL/min with a gradient of A (0.1% formic acid in 100% LC-MS grade water) and B (0.1% formic acid in 100% acetonitrile) as follow: 2% B for

2 min, 2% to 35% B in 11 min, 35% to 90% B in 2 min, hold at 90% B for 1 min, then back to 2% in 0.5 min. The QE-HF was run in a data-dependent acquisition mode triggering on peptides with charge states 1 to 3 using a mass range of 100 to 1000 m/z at 60,000 resolution, with an AGC target of 3e6 and a maximum ion time of 50 ms. The isolated ions were further fragmented by HCD using isolation window of 1.6 m/z and scanned at a resolution of 15,000.

The acquired data from the HILIC and RP separations were analyzed separately using Progenesis QI version 2.4 (Waters, Milford, MA). The chromatograms were aligned, and the peaks were detected using a comprehensive set of algorithms, including isotope and adducts deconvolution for a more accurate quantitative analysis. The library search used was NIST MS/MS version 1.0. The compounds were filtered using a score of at least 30, a mass accuracy <5 ppm and isotopic similarity of at least 90. In addition, the RP data was also analyzed using Proteome Discoverer 2.4 (Thermo Fisher Scientific, Waltham, MA). The database search was done using Mascot 2.6.2 with no enzyme specificity and Uniprot_UP000000226_Phaseolus. Mascot was searched with a fragment ion mass tolerance of 0.060 Da and a parent ion tolerance of 10.0 PPM. Peptide validation were done by Percolator with a 0.01 posterior error probability (PEP) threshold. The data were searched using a decoy database to set the false discovery rate to 1% (high confidence). The peptides were quantified using the precursor abundance based on intensity. The peak abundance was normalized for differences in sample loading using total peptide amount. The normalization factor used is the factor of the sum of the sample and the maximum sum in all files.

3.2.11 Total phenolic content (TPC) assay using LC-MS

The phenolic compounds that were targeted for the assay wee Apigenin, Caffeic acid, Catechin, Chalconaringenin, Chlorogenic acid, Cinnamic acid, Cyanidin, Daidzein, Delphinidin, Epicatechin, Ferulic acid, Gallic acid, Genistein, Hesperetin, Kaempferol, Luteolin, Naringenin, p-Coumaric acid, Phloretin, Proanthocyanindin A2, Procyanidin B2, Protocatechuic acid, Quercetin, Quercetin-3-galactoside, Quercetin-3-glucoside, Resveratrol, Rutin, Syringic acid, Vanillic acid. These compounds were extracted from the 20-50 mg of processed bean hydrolysates using 1 mL of 100% methanol. The samples were homogenized by adding 2 stainless steel beads (SSB 32) using the Tissue Lyser II (Qiagen) at 10 Hz for 15 mins. After centrifugation at 16,000 g, the supernatants were collected, the extraction process was repeated with the pellet with 1 mL of 100% methanol. The supernatants were pooled together, and vacuum dried down using a SAVANT speed-vac. The pellets were re-dissolved in 100 µL of 30% methanol.

For LC separation, ZORBAX Eclipse XDB C18 column (2.1 mm × 100 mm, Agilent) was used flowing at 0.4 mL/min. The gradient of the mobile phases A (2% acetic acid) and B (100% acetonitrile) was as follow: 6% B for 1 min, to 17% B in 4 min, to 20% B in 3 min, to 90% B in 8 min, hold at 90% B for 2 min, to 6% B in 1 min. The Shimadzu LC system was interfaced with a Sciex QTRAP 6500+ mass spectrometer equipped with a TurboIon Spray (TIS) electrospray ion source. Analyst software (version 1.6.3) was used to control sample acquisition and data analysis. The QTRAP 6500+ mass spectrometer was tuned and calibrated according to the manufacturer's recommendations. The phenolic compounds were detected using multiple reaction monitoring (MRM) transitions that were optimized earlier using the standards. The instrument was set-up to

acquire in positive and negative ion switching. For quantification, an external standard curve was prepared using a series of standard samples containing different concentrations of each compound.

3.2.12 *Statistical analysis*

The experimental design considered here was complete randomized design (CRD) between the 3 treatments - boiling (Trt1), HPP (Trt2), and HPP + boiling (Trt3), different response variables were the DH %, peptide content, ORAC value, and IC_{50} value of ABTS radical scavenging assay, here the experimental unit is the GNBs. The Anderson-Darling Normality Test was run before the analysis of variance to check the normality for each response variable. A simple one-way analysis of variance (ANOVA) was conducted and Tukey's Multiple Comparison Test was to evaluate the effect of different treatments and alcalase digestion. The treatments are divided in to two different levels, the first level was boiling (Trt1), HPP (Trt2), and HPP + boiling (Trt3); while the second level was alcalase hydrolysis. The reason we choose CRD because the alcalase hydrolysis is a common for all three treatments. To identify the effect of the treatments on the concentrations of peptides and phenolics One-way ANOVA was conducted. The Tukey's Multiple comparison Test was performed to determine the difference in the relative concentration of peptides and phenolic compounds between the treatments. All the statistical analysis was conducted using the GraphPad Prism Software version 8.0. All the graphs have been expressed as the mean ± standard deviation (SD).

3.3 RESULTS

3.3.1 Protein content and SDS-PAGE analysis

The protein content of the beans was found to be in the range between 18%-26% by the Lowry Protein Estimation method. Protein content was found to be highest for the cooked beans, however there was no significant differences observed in the protein content of the processed beans. As there was no difference observed, and the measurement was done in a view to do a separation of the processed beans and the alcalase hydrolysates, an average of the protein content was represented in percentage. SDS PAGE analysis of the proteins extracted from processed GNB, before alcalase digestion showed the presence of major storage proteins, Phaseolin, acrelin-4, lectin, phytahaemaglutinins, and protease inhibitors like α-amylase and trypsin inhibitors. Both processed bean isolates and alcalase hydrolysates were analyzed on the SDS-PAGE. Storage proteins were present in the processed bean protein isolates and also confirmed the absence of intact bean proteins after digestion with the enzyme alcalase (Figure 3.1). Prominent bands were observed around 40-53 kDa (Phaseolin), 29 kDa (Acrelin-4), for the protein isolates from processed beans (Garcia-Mora et al., 2016). The bands ranging from 18-25 kDa indicated protease inhibitors such as the presence of α-amylase inhibitor (18 kDa), trypsin inhibitor (20 kDa) in the processed bean isolates. Weak bands were observed at 20 kDa in the cooked-extracted sample (A-B) which corresponded to trypsin inhibitor. As we have used only freeze-dried supernatant (digested fraction) collected after alcalase digestion after centrifugation, the enzyme and most of the undigested proteins were retained in the pellet. The results indicate that the major bean proteins were extensively hydrolyzed after alcalase treatment.

3.3.2 Degree of hydrolysis and Peptide content Analysis

The *Degree of hydrolysis (*DH %) as calculated by the pH-Stat method, showed that DH % was significantly decreased after HPP pre-treatment (13.9 ± 1.2) as compared to cooked (19.5 ± 1.5) or the combined (19.6 ± 2.3) treatments (Fig. 3.2 A). However, the peptide content results revealed no significant difference between the differently processed beans after alcalase digestion (Fig 3.2 B). The peptide content of the cooked hydrolysate was 698.0 ± 42.3 µg/mg and that of HPP hydrolysate was 675.4 ± 34.0 µg/mg. The peptide content of the combined treatment hydrolysate was 641.0 ± 51.3 µg/mg.

3.3.3 Antioxidant activity by ABTS radical scavenging assay

The IC_{50} value of cooked (Trt-1), HPP (Trt-2) and HPP + cooked (Trt-3) beans were 417.8 ± 10.5 µg/mL, 411.9 ± 7.5 µg/mL, and 497.3 ± 12.1 µg/mL, respectively (Fig. 3.3A). The antioxidant activity of HPP + cooked (Trt-3) was significantly diminished than the cooked (Trt-1) or the HPP (Trt-2).

3.3.4 Antioxidant activity by ORAC

The antioxidant activity of the alcalase digested processed bean hydrolysates was measured based on the Trolox standard curve. The antioxidant activity of Trt-1, Trt-2, and Trt-3 were 370.8 ± 14.3 µmol TEAC/g, 285.3 ± 14.75 µmol TEAC/g, and 265.0 ± 20.1 µmol TEAC/g, respectively (Fig. 3.3 B). However, the antioxidant activity of HPP + cooked (Trt-3) was significantly lower than the cooked (Trt-1) or the HPP (Trt-2) hydrolysate. Also, the cooked bean hydrolysate showed significantly higher antioxidant activity than the HPP. Thus, the cooked bean hydrolysates were found to contain peptides with higher antioxidant activity compared to the other two treatments.

3.3.5 Identification and characterization of peptides

The data obtained from LC-MS/MS (Liquid Chromatography with Tandem Mass Spectrometry) were analyzed based on the intensity of the peaks obtained for different peptide sequences that were identified. The peptide sequences obtained after RP and HILIC were combined to measure the fold change of each of the peptide sequences. The individual intensity of each peptide sequence, of each technical replicate per treatment was calculated and was normalized with the average intensity of all the peptide sequences obtained for each treatment replicates. The fold change of individual peptide sequences was determined for different treatments. Thereafter, the peptide sequences with fold change greater than 1, were selected and those peptide sequences were used for comparing the effect of treatment on the peptide sequences (Table: 3.1). Multiple comparison analysis showed that there was significant difference found in the intensities of certain peptide sequences with fold change greater than 1, across the different treatments. Tripeptides produced were significantly reduced across the treatments both in terms of number and intensity of the peptides as compared to that of dipeptides. It was found that the peptide sequence Met-Asp (MD) was significantly higher in HPP hydrolysates (Trt-2) (29.5 ± 0.5) than the cooked (26.2 ± 8.6) or the combined (22.5 ± 8.6). Ile-Phe (IF) was found to be significantly reduced in cooked hydrolysates (18.4 ± 0.7) and HPP hydrolysates (18.8 ± 1.1) than the combined treatment (20.7 ± 1.1). The peptide YF was found to be significantly increased after boiling (4.7 ± 1.0) as compared to HPP (1.7 ± 0.1) or HPP-cooked (4.2 ± 2.8) samples. The other peptide sequences that were found to be significantly different in terms of fold change after processing and digestion were Ile-Ala (IA), IL (Ile-Leu), and Ile-Val-Arg (IVR). Both IL (61.1 ± 3.8)

and IVR (6.8 ± 1.3) were found to be significantly higher in the cooked than HPP (IL: 56.1 ± 0.8, IVR: 3.9 ± 0.5). The dipeptide IA was also significantly higher in cooked (Trt 1:16.61 ± 1.1) and reduced in other treatments (Trt-2: 12.5 ± 0.9, Trt-3: 15.7 ± 2.7) as observed in Table: 3.1. It was found that the intensities of peptide sequences, with 11-12 amino acid residues, were higher in each of the processed bean hydrolysates while the intensity of the 12 amino acids peptide sequence VNPDPKEDLRII was found to be the highest after each of the treatments. The intensity of this peptide was noted to be lowest for the HPP hydrolysate. However, over all there was no significant differences found in the longer peptides after processing and alcalase hydrolysis.

3.3.6 Identification of phenolic compounds after processing and enzymatic hydrolysis

The LC-MS method was used to measure the total phenolic content (TPC) present in the processed beans before and after alcalase hydrolysis. The phenolic content in processed bean samples before and after digestion were expressed in terms of ng/g. The results of showed that the range of TPC of processed beans after alcalase hydrolysis. Overall, all of the TPC was found to be higher in HPP (2134.23 ng/g) than that of cooked (1597.5 ng/g) or combined treatment (950.6 ng/g). The phenolic compound ferulic acid was significantly higher in HPP (1219.2 ± 95.5 ng/g) than cooked (1151.9 ± 97.0 ng/g) and significantly decreased after combined treatment (652.9 ± 41.0 ng/g). The other phenolic compound that was found to be in high amount was p-coumaric acid, which was lower in cooked hydrolysate (262.3 ± 14.8 ng/g) than the HPP (356.3 ± 41.6 ng/g) and further reduced after combined treatment (163.1 ± 7.7 ng/g). Rutin was found to be very low after the heat treatments (Trt 1: 4.6 ± 1.0 ng/g, Trt-3: 2.3 ± 2.7 ng/g). Other phenolic

compounds found to be different after treatments were genistein, naringenin, and vanillic acid (Table: 3.2).

3.4 DISCUSSION

Consumers today are aware of foods with health benefits and have shown a growing interest towards plant-based proteins. Common bean varieties, such as GNB, have already been regarded as nutraceutical food due to the presence of bioactive compounds such as bioactive peptides, polyphenols, oligosaccharides, resistant starch, and non-digestible fractions (Luna-Vital et al., 2015). In order to maximize the extraction and bioavailability of such bioactive compounds, various alternative technologies have come forward to enhance the health-beneficial functionalities of the food products. One such technology is high-pressure processing, which is a leading non-thermal processing technique with the potential to enhance the extractability and bioavailability of peptides produced from enzymatic hydrolysis as well as phenolic compounds (Garcia-Mora et al., 2016). The current study aimed to compare and evaluate the efficacy of two different pre-treatment methods, i.e. the conventional thermal pre-treatment (boiling) with non-thermal HPP treatment, in the release of bioactive peptides and phytochemicals exhibiting antioxidant activities from alcalase generated GNB hydrolysates. Pressurization of common beans has been shown to increase the hydration through an increase in water absorption and filling up of capillaries and spaces (Belmiro et al., 2018). This can affect the protein solubility as there may be more interaction of water molecules with the hydrophilic peptides present in proteins. The application of high pressure can disrupt covalent bonds like peptide bonds and disulfide bonds. Disulfide bonds are specifically involved in the aggregation that takes place on the application of high-pressure on the

food protein (Messens et al., 1997). This could be seen in Fig. 3.1, as the intensity of the soluble protein bands for the HPP-treated samples were found to be a little higher than non-HPP treated, cooked samples in the SDS-PAGE analysis. HPP induces protein-aggregation, which decreases the hydrolytic yield (Zhang et al., 2012), however, our results did not suggest any occurrence of insoluble protein aggregation at the pressure level assessed. The protein profile of the differently processed beans was almost similar, suggesting that high pressure application did not induce any change in the protein profile. Moreover, enzymatic hydrolysis of the processed beans with alcalase was shown to completely degrade majority of the proteins. Even though phaseolin had been shown to be resistant to enzymatic hydrolysis, because of its glycosylated and compact structure (Mojica & de Mejía, 2015), the degradability of the proteins after alcalase hydrolysis suggested the suitability of this particular enzyme for the generation of the GNB hydrolysates.

In terms of hydrolytic yield, the DH of cooked beans were found to be higher than the HPP-treated beans (Fig. 3.2 A). Similarly, the DH of HPP-cooked treatment was also higher than only HPP treated beans, which suggested that heat pre-treatment was more efficient in the release of peptides during alcalase hydrolysis. This is because heat leads to partial denaturation of proteins and exposes the hydrophobic residues of globular proteins, thereby increasing the accessibility of enzymes for hydrolysis (Luna-Vital et al., 2015). HPP has been reported to enhance proteolysis (Zhao et al., 2017). Even though studies have shown that HPP treatments also leads to exposed hydrophobic residues, in our case we did not see comparable DH in case of HPP treated bean hydrolysis. One probable reason for that could be the use of very high pressure of 600 MPa for the pre-

treatment. Studies have shown that HPP-assisted alcalase hydrolysis at 200 MPa produced hydrolysates containing lower content of peptides lesser than 3 kDa as compared to those produced at 100 MPa. It also showed that use of higher pressures of 400 and 600 MPa led to production of hydrolysates with higher molecular weight peptides (Chao et al., 2013; Garcia-Mora et al., 2016). Thus, the DH values were less as compared to the heat pre-treatment method as many high molecular weight peptides might not have been hydrolyzed. Furthermore, the peptide content of all the treated beans were not found to be significantly different (Fig. 3.2 B), which could be due to the sensitivity limit of the fluorometric peptide assay. Alcalase hydrolysis of bean cultivars have been shown to produce antioxidant peptides with TEAC values ranging from 1.90 to 3.78 mM mg^{-1} inhibiting ABTS (Valdez-Ortiz et al., 2012). It has been found that the antioxidant activity of peptides has been increased in the presence of aromatic amino acids such as Tyr, Trp, and Phe, as well as basic amino acids such as Arg and His, as the indole, imidazole, and phenolic groups present in these residues can act as proton donors to the needful radicals in order to sustain their molecular stability (Sarmadi & Ismail, 2010). It has also been shown that peptides with antioxidant activity consist of hydrophobic amino acids such as Leu, Val, Ala or Ile at the N-terminal (Guo et al., 2009). Furthermore, molecular mass determination of the peptides from the bean hydrolysates with antioxidant properties suggests that most antioxidant peptides are smaller in size (less than 1 kDa) with a greater chance to contain the mentioned amino acid residues at the N- and C- terminals (Luna-Vital et al., 2015). In view of this, the peptide profile analysis of our processed hydrolysates showed higher number of smaller peptides (dipeptides and tripeptides) in case of heat processed hydrolyzed beans as

compared to HPP-only, which also correlates with the higher antioxidant activity seen in the case of heat-treated bean hydrolysates. Dipeptides IA (Ile-Ala), YF (Tyr-Phe), IL (Ile-Leu), IF (Ile-Phe), and tripeptide IVR (Ile-Val-Arg) were significantly higher in cooked hydrolysates than Trt-2 and Trt-3. As per the results obtained by the ORAC Assay (Figure: 2.3 A) imply that YF may be playing a significant role in the increased antioxidant activity shown by the cooked been hydrolysates. If the N-terminal of a particular peptide sequence has a low isoelectric point, hydrophobic amino acid residue like Ala, Val, Gly or Leu, then the peptide is believed to exhibit very strong antioxidant activity (Deng et al. 2019). The specific peptide sequences that were found to be higher in boiling having Ile (IL) on its C-Terminal and Leu (L), Phe (F) and Ala (A) on the N-terminal may have contributed towards the enhance antioxidant activity of the cooked hydrolysates. Tripeptide IVR (Ile-Val-Arg) is also increased in cooked samples and may be aiding towards the antioxidant activity (Table: 3.1). Furthermore, longer peptides (> 5 amino acid residues) were also identified by the LC-MS/MS data. However, there was no statistically significant differences found in the abundance of longer peptides across the treatments except for identifying the 12 amino acids sequence, VNPDPKEDLRII, to be present in abundance for each of the treated hydrolysates. Lastly, the presence of phenolic compounds was also assessed for all the processed bean hydrolysates and it was found that in case of heat-treated beans, the TPC was significantly diminished than after HPP-treatment (Table: 3.2). This is in accordance to literature, where, heat treatments have been shown to reduce phenolic contents in beans (Wang et al., 2009). The positive effect of HPP treatment on TPC is due to the disruption of protein-polyphenol and protein-protein non-covalent interactions (Garcia-Mora et al., 2016). All the phenolic

compounds that were detected in higher amounts in HPP hydrolysates. Rutin was probably most effected after heat treatments than after HPP (Table: 3.2). Vanillic acid was not detected after combined treatment and alcalase hydrolysis. The increased amount of ferulic acid in the HPP-treated bean hydrolysates might be due to HPP assisted release of such hydroxycinnamic acids which were otherwise non-extractable. Ferulic acid has been shown to have antioxidant activity as well due to high free radical scavenging, singlet oxygen quenching capacity, and transition metal chelating properties (Craft et al., 2012).

The major findings of this study were that the accessibility for digestive enzymes was reduced after HPP-pretreatment of GNB, antioxidant activity was reduced after HPP as well as after combined treatments and enzymatic hydrolysis. Besides, these two key findings, the variations in phytochemicals after different treatments and hydrolysis has aided to determine the more efficient pre-treatment. As found in previous literature, our results also suggested that cooking and enzymatic hydrolysis enhanced antioxidant activity of the beans (Ketharin et al., 2019). HPP at 600 MPa for 5 mins, did not enhance the accessibility of the protease to cleave proteins and this could be due to specific limitation in selecting specific pressure and duration of application. The pressure used and the duration of application could be optimized further to see if the degree of hydrolysis could be enhanced after HPP pre-treatment as reported (Zhao et al., 2017).The results obtained from TPC analysis, suggested that the phytochemicals naturally present in the beans was not affected by the HPP but was damaged by heat treatments. Overall, we can conclude that although HPP along with enzymatic hydrolysis did not enhance

release of peptides with antioxidant activity, HPP is preferred over cooking in maintaining the stability of the phenolic compounds present in the beans.

3.5 CONCLUSION

GNB is a promising protein source to derive bioactive peptides by enzymatic hydrolysis. Boiling can affect the release of peptides with antioxidant activity but also destroy the phytochemicals resent in beans. On the other hand, High Pressure processing is being studied as an alternative to boiling to release peptides with antioxidant activity, however HPP treatment did not resulted higher antioxidant activity compare to boiling but it was helpful in maintaining the stability of the phenolic compounds that could be lost due to thermal processing. We hence conclude from the results obtained that there was a significant difference between HPP and cooked beans in terms of the DH. The peptide content did not have any significant effect due to processing. The measurement of antioxidant activity by the ABTS method revealed that after combined processing and digestion, the antioxidant activity was significantly reduced. Processing as well as alcalase digestion cumulatively have an impact on the antioxidant activity of the peptides derived from GNB. However, the antioxidant activity as measured by ORAC showed that the cooked beans retain better antioxidant activity after hydrolysis with alcalase as compared to HPP or combined-processed beans. The enhanced antioxidant potential of the cooked hydrolysates is influenced by the presence of some phenolic compounds and unique peptide sequences. The differences in the intensity and number of peptide sequences in the processed bean hydrolysates was an important factor that correlates to the differences in the antioxidant activity of cooked beans than the HPP samples. The

stability of the phenolic compounds was enhanced in HPP hydrolysates. While phenolic compounds were found to be reduced for the heat processed hydrolysates, it was found to be more stable after HPP as well as alcalase hydrolysis. The synergistic effect of all the peptides present in higher intensity in the cooked hydrolysates contribute to the better antioxidant activity compared to the HPP hydrolysates. Cumulatively, it can be concluded that boiling is still considered to be the preferred than High Pressure processing (HPP) in terms of the release of peptides with antioxidant activity.

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FIGURES AND TABLES

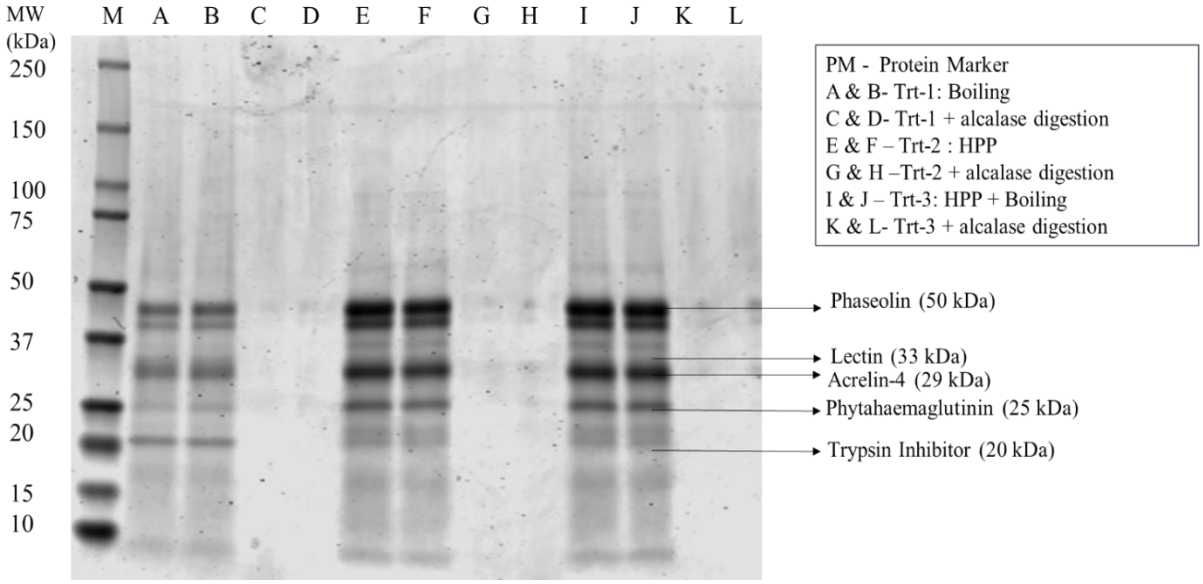

Figure 3.1. SDS-PAGE Analysis of processed GNB and Processed bean hydrolysates.

SDS-PAGE analysis of processed Great Northern Beans using a 4-20% precast polyacrylamide gel, 30 µg of protein (15 µL/well) was loaded from a stock solution of 12 mg/mL of the sample. The prominent bands for phaseolin (50 kDa), acrelin-4 (30-40 kDa), and phytohaemaglutinins (20-25 kDa) were observed after boiling and high pressure processing (HPP) in lanes A, B, E, F and I, J. Alcalase hydrolysates show no prominent bands on lanes C, D, G, H, K and L. Molecular weight of peptides were lower than 10 kDa, hence prominent bands were not observed on the SDS-PAGE.

Note: 12 mg/mL was diluted thrice to 4 mg/mL, 2 X loading dye was added to dilute it to 2 mg/mL or 2 µg/µL. On loading 15 µL/ well of sample has 15 x 2 = 30 µg of sample.

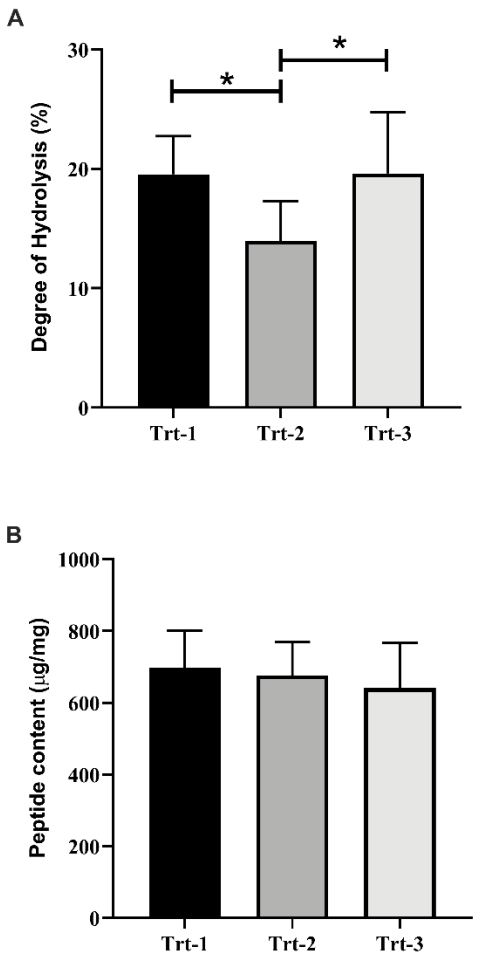

Figure 3.2. (A) Degree of hydrolysis and (B) peptide content of processed beans after alcalase digestion. Beans were processed as follows: (Trt-1) boiling, (Trt-2) high pressure processing, and (Trt-3) boiling after HPP. Error bars represent Mean and Standard Deviation (SD). Asterisks show difference in means (*, $p < 0.05$; **, $p < 0.01$).

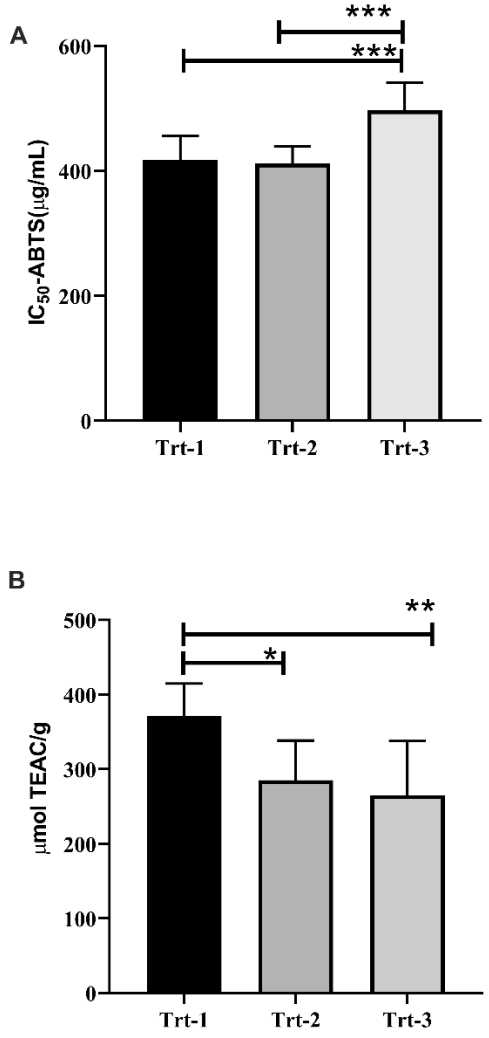

Figure 3.3. A. Antioxidant activity of processed whole bean hydrolysates by ABTS Radical scavenging method. Error bars represent Mean and Standard Deviation (SD), Asterisks show difference in means (*, $p < 0.05$; **, $p < 0.01$). **B.** Antioxidant activity of processed whole bean hydrolysates by Oxygen radical absorbance capacity (ORAC). The statistical comparison was conducted between treatments. Error bars represent Mean and Standard Deviation (SD)*statistically significant difference ($p<0.05$).

Table 3.1: Effect of Treatments on the generation of Dipeptides and Tripeptides

Treatments	Peptide Sequences (fold change)					
	MD	YF	IA	IL	IF	IVR
Trt-1	26.2 ± 8.6^{b}	4.7 ± 2.0^{a}	16.6 ± 1.16^{a}	61.1 ± 3.8^{a}	18.5 ± 0.7^{b}	6.9 ± 1.3^{a}
Trt-2	29.5 ± 0.5^{a}	1.7 ± 0.1^{b}	12.5 ± 0.9^{b}	56.1 ± 0.8^{b}	18.8 ± 1.1^{b}	3.9 ± 0.5^{c}
Trt-3	22.5 ± 8.6^{c}	4.2 ± 2.8^{a}	15.7 ± 2.7^{a}	60.1 ± 5.5^{a}	20.7 ± 1.1^{a}	4.2 ± 1.1^{c}

Note: The fold change in the relative intensity of the peptide sequences was expressed as Mean ± SD. Each column (peptide wise) grouping indicates the effect of treatment, a is the highest amount (relative intensity) of peptides present, followed by b and c.

Table 3.2: Effect of Treatments on the Phenolic compounds present in

Treatments	Phytochemicals (ng/g)				
	p-Coumaric acid	Ferulic acid	Genistein	Naringenin	Rutin
Trt-1	262.3 ± 14.8^{b}	1151.9 ± 97.0^{b}	42.6 ± 12.5^{b}	36.8 ± 2.7^{b}	4.6 ± 1.0^{b}
Trt-2	356.3 ± 41.6^{a}	1219.2 ± 95.5^{a}	108.0 ± 17.2^{a}	144.5 ± 29.7^{a}	103.1 ± 22.0^{a}
Trt-3	163.2 ± 7.7^{c}	652.9 ± 41.0^{c}	48.3 ± 13.3^{b}	34.7 ± 4.1^{b}	2.3 ± 2.7^{b}

Note: The amount of phenolic compounds present after each treatment, were expressed as Mean ± SD. Each column (phenolic compound wise) grouping indicates the effect of treatment, a is the highest amount (relative intensity) of peptides present, followed by b and c.

CHAPTER 4
CONCLUSION

The motivation behind this research is to focus on the possibility of High pressure processing to be as efficient as boiling process. Hence, the null hypothesis was framed such that HPP pre-treatment along with enzymatic hydrolysis will be an effective alternative strategy than boiling to enhance antioxidant activity of beans. However, the alternative hypothesis revolves around choosing boiling pre-treatment along with enzymatic hydrolysis to be more effective than the HPP pre-treatment in enhancing the antioxidant activity of the Great Northern beans (GNB).

According to the results obtained in this study, HPP did not denature the storage proteins present in the GNB as observed on the SDS-PAGE. The results also suggested that cooking (Trt-1) showed a higher degree of hydrolysis which was decreased after HPP treatment, suggesting that the high-pressure treatment diminished the enzyme accessibility to cleave GNB proteins in order to release bioactive peptides. Different pre-treatments before the hydrolysis of beans, however, did not affect the peptide content of the processed whole bean hydrolysates. Antioxidant activity was modulated after HPP and alcalase digestion. From the results of *in vitro* antioxidant activity assays, we found that cooking and HPP showed similar antioxidant activity as measured by the ABTS method. However, boiling was proven to have enhanced antioxidant activity than HPP pre-treatment as measured by ORAC. As phenolic compounds present in GNB could be an important marker for the antioxidant activity, the TPC analysis was performed by the LC-MS targeted assay. The results suggested that HPP facilitated in maintaining the stability of phenolic compounds rather than degradation or loss that was observed after

the thermal treatments and alcalase hydrolysis. The LC-MS/MS peptide identification and quantification indicated a significant increase in a few dipeptides and tripeptides in the cooked bean hydrolysates that could potentially contribute towards the observed antioxidant activity. To summarize, from this study it's noted that the conventional process of cooking is by far better than HPP to derive bioactive peptides from GNB with higher antioxidant activity. In other words, based on the results observed in the study, we reject the stated hypothesis and accept the alternative hypothesis that suggests boiling is preferred over HPP to release of peptides showing antioxidant activity. However, HPP pre-treatment retained the stability of phytochemicals in contrast to the degradation of phytochemicals after heat treatments. There was a limitation in this study, and that is about the selection of the pressure and its duration, an optimization study could be done in the future to identify if the release of the peptides were enhanced by pressure conditions used and for different span of time.

However, the results obtained entail further, extensive research to be validated by *in vitro* cell culture studies and some *in vivo* studies as well. It is essential, to prove if there is any modulation in other biological activities, exhibited by the bean derived peptides, in order to confirm the peptides from beans to be health-promoting by ameliorating the risk of many of the chronic diseases. Besides this, literature suggests that growing conditions during the cultivation of beans could affect the protein content as well as the antioxidant activity of the beans. Hence, for the future prospect on the study on GNB, it is proposed that the effect of growing conditions could be studied extensively on the release of peptides with antioxidant activity. As cooking and simulated gastrointestinal digestion procedures mimic the basic method the beans as consumed by people, Figure: 4.1 shows

a preliminary schematic about the flow of experimental design that could be followed to work on this proposed future project work.

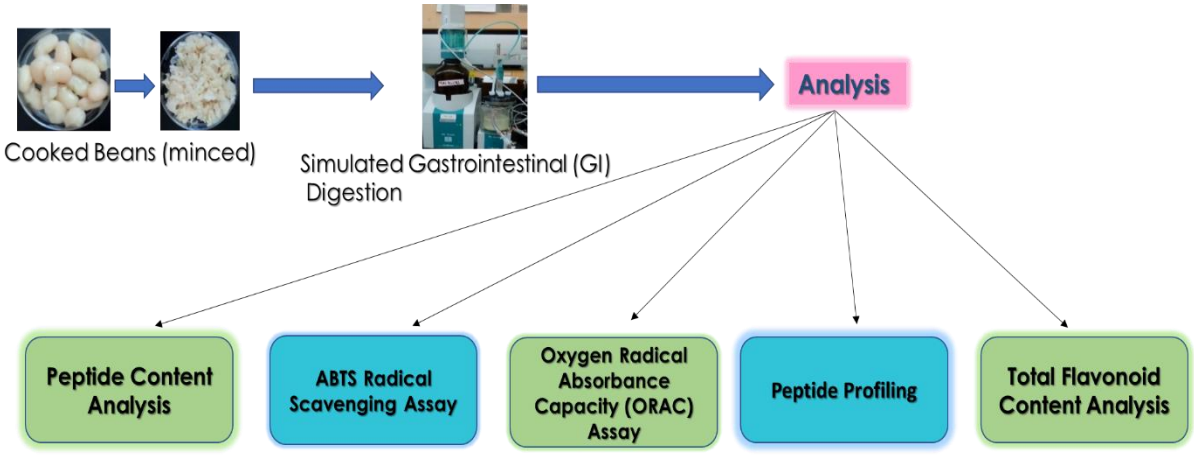

Figure 4.1: Flow Chart showing the Experimental Design on Future Project.