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Studies On The Physicochemical Characterization Of Flours And Protein Hydrolysates From Common Beans

Hollman Andres Motta Romero
University of Nebraska-Lincoln, hollmanmoro@huskers.unl.edu

STUDIES ON THE PHYSICOCHEMICAL CHARACTERIZATION OF FLOURS AND PROTEIN HYDROLYSATES FROM COMMON BEANS

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Hollman Andres Motta Romero

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Hollman Andres Motta Romero, M.S.

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Advisor: Yue Zhang

Pulses represent a rich source of nutrition and have several potential health benefits such as the reduction of the risk of developing cardiovascular diseases, obesity, and diabetes. Beans from the species *Phaseolus vulgaris* and *Cicer arietinum* are important crops raised in some regions of the United States, including Western Nebraska. Nevertheless, the traditional preparation procedures involved in the cooking of these beans are tedious and time-consuming, thus leading to the underutilization of these resources. Therefore, the present work focused on evaluating alternative utilizations of these beans in order to improve their economic value.

One alternative to improve the consumption of pulses is the utilization of bean flours. The study of physicochemical properties of these flours and the effect of their major components on their functional characteristics of flours is essential for the development of food products made with bean flours. Chapter two dealt with the physicochemical characterization of starches from great northern, navy, red kidney (*Phaseolus vulgaris* L.), and garbanzo beans (*Cicer arietinum* L.) and studied the effect the starch fraction on the rheological behavior of flours during thermal treatment. Our results showed that the pasting properties of flours were significantly affected by the amylose content and granule size of

starches. It was also found that garbanzo and navy bean develop stronger networks than great northern or red kidney bean flours and that these two flours may serve as alternative ingredients for gluten-free products such as pasta.

Since dry beans are rich in protein, the utilization of bean protein concentrates as food ingredients such as emulsifiers might be promising. To compete with commercial protein ingredients, the functional properties of bean concentrates should be further improved. In chapter three, the effects of controlled enzymatic hydrolysis on the functionality of great northern and navy bean protein concentrates were studied. Our results suggested that controlled hydrolysis of these proteins with alcalase or papain significantly improved their functional properties and that protein hydrolysates from *Phaseolus vulgaris* beans might have potential use in the food industry as emulsifiers.

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Organization of the manuscript

This manuscript is composed of three chapters. The first chapter walks the reader through an introduction to the concepts to be further investigated in the following chapters. The second chapter focuses on the physicochemical characterization of flours and starch-rich fractions from pulses. Whereas, chapter three, deals with the study of controlled enzymatic hydrolysis as an alternative to improve the emulsifying properties of beans commonly grown in Nebraska.

The second and third chapter will be submitted for publication to *Food Chemistry* and *Journal of Agricultural and Food chemistry*, respectively. Therefore, these have been formatted according to the guidelines of each journal. The references, tables, and figures belonging to each chapter are added at the end of each section.

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CHAPTER 1. INTRODUCTION

1.1. Introduction to pulses

Pulses are the dry edible seeds of plants belonging to the Fabaceae family. Due to their adaptability to a wide range of climatic and soil conditions and the fact that these crops serve as host for nitrogen-fixing bacteria (Peralta et al., 2016), these have been grown throughout the United States. Currently, the United States is the third largest producer of edible beans only behind Myanmar and Brazil (Statista, 2018). These beans may play an important role in the economy of some regions of the country, such as Western Nebraska, where the climate is arid. According to the USDA, around 3,500 million pounds of pulses were produced in 2017, and the exports were valued over 450 million dollars (USDA, 2018), with North Dakota, Michigan, and Nebraska ranked within the top three largest producers.

The consumption of pulses shows several potential health benefits such as the reduction of the risk of developing cardiovascular diseases, obesity, and diabetes (Bazzano et al., 2011; Mattei, Hu & Campos, 2011). However, the per capita consumption by the American population has historically remained as low as 3.5 kilograms per person per year (USDA, 2018), which is low when compared to regions such as South America where up to 10 kg are consumed per person (Leterme & Muños, 2002).

The tedious and time-consuming cooking procedure of *Phaseolus vulgaris* and *Cicer arietinum* beans might be the main factor hampering their popularity among the American population. Traditionally, these beans are soaked for long periods (8-12 hours) to rehydrate the seeds and shorten their cooking time. However, long cooking times (40-60 minutes) are still recommended for most beans after soaking (Borchgrevink, 2012). Furthermore, when stored under unfavorable conditions, beans might develop the hard-to-cook defect which results in the inability of the seeds to soften sufficiently even after a long period of soaking and cooking (Yi et al., 2016). Therefore, there is a need to seek for alternative uses of pulses in order to improve their utilization and consumption.

1.2. Utilization of pulses flours

One strategy to promote the consumption of pulses is the utilization of their flours. Pulses flours are rich in protein with contents ranging from 20 to 30% (dry basis) (Ai et al., 2017; Du et al., 2014). Additionally, these flours are rich in vitamins, mineral, and amino acids which may be deficient in cereal flours (Mitchell et al., 2009; Nosworthy et al., 2017). Therefore, different pulse flours have been combined with cereal flours to enhance the nutritional quality of products such as pasta or extruded snacks. For instance, the protein and fiber contents in semolina pasta have been increased by the incorporation of bean flour (Gallegos-Infante, Rocha-Guzmana, Gonzalez-Laredo, 2010; Petitot et al., 2010). Nevertheless, some detrimental effects on the cooking and textural quality of final goods (i.e., higher cooking loss and lower hardness) may be caused by

the addition of non-gluten proteins which might weaken the structure of pasta dough (Petitot et al., 2010). Similarly, the incorporation of pinto bean (*Phaseolus vulgaris* L) flour into brown rice extruded snacks increased their protein content, decreased the starch digestibility, but negatively affected physical attributes such as hardness, density, and expansion (Sumargo et al., 2016).

Understanding the physicochemical properties of pulses flours is imperative to facilitate their efficient application, either into composite flours or by themselves. The functional properties of a wide variety of bean flours including black, great northern, navy, red kidney (*Phaseolus vulgaris* L.) (Ai et al., 2017), and garbanzo bean (*Cicer arietinum* L.) (Du et al., 2014; Kaur & Singh, 2005) have been evaluated and large variability has been found among varieties and genera. For instance, flours from some varieties of navy and pinto beans have developed stronger structures than great northern bean flours when used to develop baked goods (Ai et al., 2017). Additionally, it has been found that chickpea flours present lower resistance to shear at high temperatures and lower tendency to retrogradation when compared to *Phaseolus vulgaris* beans such as pinto or red kidney (Du et al., 2014).

Since pulses are gluten-free crops and present high contents of starch (22-45%, dry basis) (Hoover et al., 2010), the physicochemical behavior of whole bean flour might depend on the functional and chemical properties of their starch fraction. Although the physicochemistry of bean starches have been widely studied (Hoover et al., 2010), and it has been reported that starch properties

such as granule size and morphology affect the cooking quality of the whole been seeds (Sharma et al., 2015), the relationship between the starch properties and the properties of pulse flours has not been studied.

1.3 Pulse Starches

To understand the behavior of starch under cooking conditions, its pasting properties are often studied using the Brabender viscoamylograph (BVA) method which assesses the viscosity of starch slurries subjected to a programmed heating and cooling cycle (Rui & Boye, 2013). Upon heating in excess of water, starch granules swell water and increase their volume to several times their original size, thus increasing the viscosity of the starch slurry. The structural integrity of these swollen granules is susceptible to shear forces and high temperatures, hence, after reaching a maximum viscosity, the granular structure of starch is disrupted by shear, which results in a decrease of viscosity and extensive leaching of amylose from the granules (Bemiller & Whistler, 1996).

Upon cooling, the leached-out polymers re-associate and form junction zones, leading to increases in viscosity in a process referred to as retrogradation (Bemiller & Whistler, 1996). Consequently, the pasting parameters of starch including pasting temperature, peak viscosity, breakdown, set back and final viscosity could be observed in a Brabender viscosity curve (Fig. 1.1) (Schoch & Maywald, 1968).

Compared to cereal starches, pulse starches generally show a distinctive pasting behavior. Due to their high amylose content, pulse starches present

stability to shear and high temperatures, which is evidenced by the absence of a breakdown viscosity in the Brabender viscosity curve (Fig. 1.1) (Hoover et al., 2010; Schoch & Maywald, 1968). The high amylose-amylopectin ratio of pulse starches is also known to limit their swelling capacity (Hoover et al., 2010) and to decrease their digestibility (Rebello, Greenway & Finley, 2014).

Besides its chemical composition, the morphology and size of starch granules are also known to significantly affect the cooking properties of starch (Lindeboom, Chang & Tyler, 2004). The starch granules from *Phaseolus vulgaris* L (Ovando-Martínez et al., 2011) or *Cicer arietinum* L (Hughes et al., 2009) seeds are commonly reported to be oval or spherical, with smooth surfaces, and size distributions within the 5-40 μm range (Hoover et al., 2010), whereas starches from cereals such as wheat or rice tend to be composed of smaller granules (Lindeboom, Chang & Tyler, 2004). Generally, the gelatinization properties of starch are related to the size that small granules tend to have a lower gelatinization temperature than large granule starch, (Lindeboom, Chang & Tyler, 2004). Therefore, it is plausible that the variability of the starch physicochemistry among different pulses could significantly dictate the cooking behavior of pulse flours. For instance, the retrogradation degree and the ability to form strong viscoelastic gels (high storage modulus) of rice flour have been improved by partial replacement with mung bean (Fengfeng, Yaping, Na, Han, & Xueming, 2015). The inclusion of mung bean starch into this rice composite flour improved the cooking and textural quality of noodles by decreasing cooking loss

and increasing elasticity and firmness. These improvements were attributed to the high amylose content of mung bean starch, its restricted swelling capacity, and ability to form stronger network structure than rice flour by itself (Fengfeng, Yaping, Na, Han, & Xueming, 2015).

Unfortunately, the relationship between starch properties and the physicochemical behavior of whole bean flours has been rarely studied, which may provide deeper insight into the variability of flour functionality and is meaningful for the development of bean flour-based food applications.

Besides the utilization of whole bean flours, the fact that some pulses such as those from the *Phaseolus vulgaris* L. species are wildly grown in the United states and are rich sources of proteins, highlights the potential of their protein fraction to be utilized as ingredients by the food industry.

1.4. Dry bean proteins as alternative functional food ingredients

1.4.1. Valuable uses of dry bean proteins, opportunities, and challenges

Since proteins from animal resources such as milk and egg generally present superior functional properties than plant proteins, these have been widely used as commercial food ingredients such as foaming and emulsifying agents by the food industry. However, critical factors such as the rapid growth of the world's population, the large greenhouse gas emissions related to the production of animal proteins (Herrero et al., 2013), and moral concerns from consumers demand the food industry to explore alternative protein sources. Plant

proteins, from the Fabaceae family, might represent a sustainable and cost-effective protein source.

In a wide range of applications, emulsification is one of the most important functional properties of food protein ingredients. Due to their amphiphilic nature, plant proteins can stabilize oil-in-water emulsions by adsorbing onto the surface of the oil droplets to lower their interfacial tension (McClements, 2004). However, when compared to animal proteins such as β-lactoglobulin, plant proteins present inferior emulsifying properties, as evidenced by larger droplet size and lower droplet surface charge of oil-in-water emulsions (Benjamin et al., 2014). Therefore, the limited functionality of plant proteins is the main obstacle towards their widespread utilization.

The emulsifying properties of plant proteins are thought to be dependent on their ability to diffuse from the bulk solvent to the oil-water interface and to subsequently undergo structural rearrangements to maximize the thermodynamically favorable interactions between the protein and the polar and nonpolar phases (Dickinson, 2010; McClements, 2004). The diffusion and adsorption rates of protein onto the interface might be dependent on their molecular size, while the ease of structural rearrangement is dependent on the protein structure flexibility. It has been shown that non-globular and unstructured (rich in random coils) proteins rapidly adsorbs onto the interface and immediately attain an interfacial tension equilibrium, while globular proteins (most plant proteins) present a more time-dependent interfacial behavior due to the time

required to unfold their secondary and tertiary structure (Liang & Tang, 2013; Wilde, 2000).

Additionally, the stronger electrostatic repulsion forces occurring between oil droplets of emulsions stabilized by animal proteins has been reported to also contribute to their superior emulsifying properties (Joshi et al., 2012; Ricky, Lam & Nickerson, 2013). For these reasons plant proteins from sources such as lentil, soybean, pea and rice are clearly outperformed by animal proteins (Joshi et al., 2012; O'Sullivan et al., 2016).

Therefore, the limited functionality of plant proteins calls for further research on functional improvements to make these proteins more attractive to the food industry. The alternation of protein functionality through physical treatments such as heating, high pressure, or ultrasound (O'Sullivan et al., 2016), and chemical treatments such as glycosylation (Lui et al., 2012), deamidation, and acylation (Wanasundara, & Shahidi, 1997) have been investigated. However, the requirement of specialized equipment, exposure of proteins to harsh conditions, and the formation of harmful by-products discourage the practical applications of some of these methods. On the other hand, since controlled enzymatic hydrolysis can be performed without specialized equipment, it might be a promising alternative for modifying the functional (Wouters et al., 2016a) and biological (Luna-Vital et al., 2015) properties of bean proteins.

1.4.2. Enzymatic hydrolysis to improve the functionality of dry bean proteins

Although there is limited research on the effect of enzymatic hydrolysis on the functional properties of proteins from *Phaseolus vulgaris* beans, enzymes such as pepsin, pancreatin, alcalase, and papain have been utilized to treat proteins from kidney and black beans. Wani et al., 2015 reported the improvement of protein solubility, oil and water absorption capacities, and the emulsifying properties of kidney bean protein isolates when treated with papain. Similarly, Evangelho et al., 2017 found that emulsions prepared with black bean protein hydrolysates produced with alcalase were more stable than those stabilized by pepsin-produced hydrolysates or the black bean protein isolates.

Solubility is one of the most important functionalities of proteins, which may significantly affect the emulsifying properties. Usually globular and poorly charged plant proteins present poor solubility (Damodaran, 1996). Therefore, treatments are required to improve their water solubility, which results in higher emulsifying and foaming properties. Since enzymatic hydrolysis yields peptides with lower molecular sizes and exposes ionizable amino acids previously buried in the native protein structure, hydrolysates might present better solubility than native proteins. A large variety of enzymes including alcalase (Jamdar et al., 2010; Lin et al., 2016), chymosin (Barać et al., 2011), pepsin, trypsin (Wouters et al., 2016b), and papain (Wani et al., 2015), among others, have been used to produced hydrolysates from plant proteins with generally better solubility than

native proteins, especially at the protein isoelectric point (Drago & González, 2000).

Enzymatic hydrolysis might be an effective tool to obtain surface active peptides from plant proteins because it yields shorter peptides with faster diffusion rates to the interface and promotes their absorption to the interface by exposing hydrophobic amino acid clusters to the peptide surface (Wouters et., 2016a). For instance, hydrolysates from common beans (Wani et al., 2015; Evangelho et al., 2017; Betancur-Ancona et al., 2014), peas (Barać et al., 2011), and chickpea (Yust et al., 2010) have presented better emulsifying properties than the initial protein concentrates. Nevertheless, the utilization of one specific enzyme does not always result in functionality enhancement. While trypsin was an appropriate enzyme to treat gluten (Drago & González, 2000), it detrimentally affected the emulsifying properties of lentil protein concentrates (Avramenko, Low, & Nickerson, 2013). Similarly, alcalase improved the functionality of chickpea (Yust et al., 2010) but was not appropriate to treat peanut protein concentrates (Jamdar et al., 2010). Although it has been demonstrated that papain and alcalase are good choices to treat protein concentrates from *Phaseolus vulgaris* L. varieties such as red kidney and black bean, their suitability to treat proteins from bean varieties largely grown in Nebraska such as great northern or navy bean, to the best of our knowledge, has not been studied before.

Although enzymatic hydrolysis could be an effective tool to improve the utilization of bean proteins, most of the work published up-to-date limited their scope to compare the functional properties of hydrolysates and native proteins, while the causes of functionality improvement have not been adequately investigated to some extent. Therefore, as the second objective of this research, we aimed to explore the structure-function relationship of hydrolysates from *Phaseolus vulgaris* beans for a more comprehensive understanding of the characteristics of peptides responsible for their enhanced functionality.

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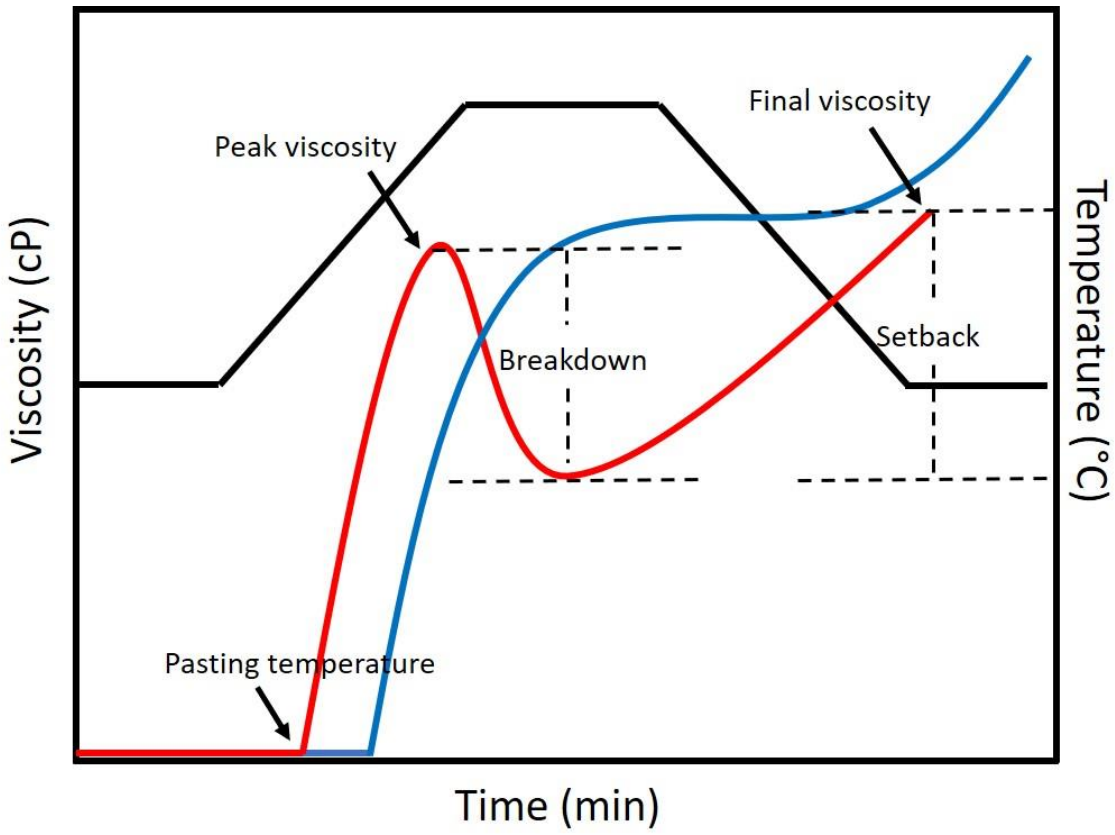

Figure 1.1. Typical Brabender viscosity curve of starch dispersions.

CHAPTER 2. PHYSICOCHEMICAL PROPERTIES OF STARCH FROM FOUR BEAN VARIETIES AND THEIR RELATIONSHIP WITH THE RHEOLOGICAL BEHAVIOR OF FLOUR AND PASTA QUALITY

2.1. Abstract

The rheological behavior of flour dispersions from four popular dry bean market classes including great northern, navy, red kidney (*Phaseolus vulgaris* L.), and garbanzo beans (*Cicer arietinum)* were evaluated, and compared to soft wheat as a gluten-containing control. Their starches were isolated and the relationships between flour behavior and starch characteristics were studied. Soft wheat (56%) and garbanzo (39%) flours presented higher starch contents than the *Phaseolus vulgaris* beans (33-35%), which resulted in the development of stronger gel network structures. It was found that starch amylose content and granule size affected the swelling and pasting properties of both starches and flours. Rheology tests suggested that garbanzo and navy bean flours developed the strongest structures among the gluten-free samples included. Therefore these were chosen to develop gluten-free pasta. The cooking and texture parameters of these pastas were also studied and compared with soft wheat pasta.

2.2. Introduction

Dry beans are the dicotyledonous seed of plants belonging to the Leguminosae family, which are widely cultivated all around the world due to their global adaptability and drought tolerance. Although their origin has been tracked to Central America and the Andean region of South America, the United States (US) has become one of the largest producers of dry edible beans worldwide nowadays. According to the USDA, in 2017 around 3.500 million pounds were produced in the US and the exports were valued over 450 million dollars (USDA, 2018), with North Dakota, Michigan, and Nebraska ranked within the top three producers. Among these, *Phaseolus vulgaris* L. varieties such as pinto, navy, great northern, kidney beans, and *Cicer arietinum* (garbanzo bean) are the most common market classes grown in the US.

Dry beans are rich in proteins and minerals and are considered a good source of dietary fiber (Tharanathan & Mahadevamma, 2003). In addition to their high nutritional value, some health benefits such as the reduction of the risk of cardiovascular diseases, obesity, and diabetes have been associated to their consumption (Tharanathan & Mahadevamma, 2003). However, the traditional preparation method of beans, involving both long soaking and cooking periods, is seen as tedious and time-consuming by consumers, hence leading to the underutilization of these beans. One alternative to improve the consumption of dry beans is the utilization of bean flours. For instance, bean flours have been incorporated into composite flours to increase resistant starch content and to

improve the protein quality (Sumargo, Gulati, Weier, Clarke, & Rose, 2016). Similarly, the addition of common bean (*Phaseolus vulgaris L.*) flour to semolina spaghetti decreased the starch digestibility and increased the protein, and fiber content of spaghetti (Gallegos-Infante, Bello-Perez, Rocha-Guzman, Gonzalez-Laredo, & Avila-Ontiveros, 2010). Nevertheless, cooking and sensory attributes such as cooking loss (Gallegos-Infante et al., 2010), appearance, and texture are simultaneously affected by the addition of legume flours to semolina (Bahnassey & Khan, 1986). Bean flours have also been utilized to develop gluten-free (GF) foods such as pasta in which the nutritional quality is enhanced (Giuberti, Gallo, Cerioli, Fortunati, & Masoero,2015). However, when compared to traditional wheat products, these GF goods usually show different texture and cooking quality and are significantly affected by the varieties of beans utilized (Bahnassey & Khan, 1986). Unfortunately, there is limited research on the physicochemical properties of bean flours and understanding the influence of their major components on the functional characteristics of flours, which are essential for the further development of food products made with bean flours.

Starch is one of the major constituents of beans, it represents about 22-45% of the total seed weight (Hoover & Ratnayake, 2002). Therefore, due to its presence in such large proportion, the functional behavior of bean flours might be significantly affected by starch properties such as granule morphology, size, and chemical composition. The characteristics of isolated bean starches have been previously studied (Hoover, Hughes, Chung, & Liu, 2010) and the effect of starch

composition on its functional properties has been reported. For instance, the large amylose content of legume starches results in high gelatinization temperatures, restricted swelling, and small or absent breakdown viscosity values (Ma, Wang, Wang, Jane, & Du, 2017; Schoch & Maywald, 1968). Nevertheless, the characteristics of starch have not been related to the functionality of the whole bean flours. The study of the physicochemical properties of bean flour from different varieties and the effect of their major constituents on the flour properties is essential for their efficient utilization.

Therefore, this work aimed to characterize the physicochemical properties of starches and flours from four bean varieties including three *Phaseolus vulgaris* L. beans (great northern, navy, and red kidney) and one *Cicer arietinum* (Garbanzo). The second objective was to understand the relationship between these properties of starch with the rheological behavior of whole bean flours and the quality of resultant pasta.

2.3. Materials and methods

2.3.1. Materials

Garbanzo, great northern, navy, red kidney beans, and soft wheat seeds were purchased from a local grocery and proceed into flour using a laboratory scale hammer mill and stored at 4 °C until utilization. The amylose/amylopectin assay and the total starch (amyloglucosidase/α-amylase) kits were purchased from Megazyme International (Megazyme, Wicklow, Ireland). Other reagents such as sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ).

2.3.2. Characterization of flours

2.3.2.1. Proximate composition of flours

Total starch, crude fat and ash content of flours were determined in triplicate following the AACCI 76-13.01 (AACCI, 1995), AACCI 30-25.01 (AACCI, 1961), and AACCI 08-01.01 (AACCI, 1961) approved methods respectively. The nitrogen content in flours was determined by the Dumas combustion method using a LECO nitrogen analyzer (LECO Corp, St Joseph, MI). The protein content was determined from the nitrogen content using a conversion factor of 6.25.

2.3.2.2. Rheological properties of flours

Rheological properties of flours were measured using a MCR-301 rotational rheometer (Anton Parr, Graz, Austria) equipped with a 50 mm diameter parallel plate measuring system. The measurements were performed on flour dispersions (10%, solids). The gap size, strain, and frequency were set at 1 mm, 2 %, and 1 Hz respectively. To perform temperature sweep tests, the samples were heated from 40°C to 95°C and then cooled down to 25°C at a rate of 1 °C/min. After temperature sweep tests, samples were held 5 min at 25 °C and a frequency sweep test from 0.1 to 25 HZ was performed to study the viscoelastic properties of the samples after cooling.

2.3.2.3. Pasting properties of flour and starches

The pasting properties of bean flours and their isolated starches (section 2.3) were determined using a Brabender Micro Visco-Amylo-Graph (Brabender

OHG, Duisburg, Germany). Flour or starch dispersions (10% db; 10.0 g total weight) were equilibrated at 50°C for 1 min, heated to 95°C at a 6 °C/ min heating rate and held at this temperature for 5 min, then cooled down to 50°C at a 6°C/min cooling rate and held at 50°C for 2 min. The spindle speed was set at 160 rpm.

2.3.3. Starch extraction and characterization

2.3.3.1. Starch isolation

In brief, wheat or bean flour was mix with water in a mass ratio of 1:10. The slurry was adjusted to pH 10 using 1.0 M NaOH and stirred for 1h to solubilize proteins. Subsequently, the slurry was filtered through a 75-µm-mesh sieve to separate insoluble fiber and was centrifuged at 1600 *g* for 30 min. The aqueous phase was discarded, whereas the bottom white sediment was collected, and recovered as starch portion after washing two times with distilled water. The recovered starch was dried in a forced air oven at 50 °C overnight.

2.3.3.2. Amylose content, swelling power, and solubility of starch

Amylose content of bean starches was measured using an amylose/amylopectin assay kit (Megazyme, Wicklow, Ireland). For swelling power determination, 10 ml of 5% (w/w) starch dispersions in centrifuge tubes were heated in a water bath at 70°C for 30 min, and the tubes were periodically vortexed to prevent granules sedimentation. After heating, the samples were cooled down to room temperature and centrifuged (1600 g, 10 min). The pellet was weighted before and after drying in an oven at 100 °C and the supernatant

was collected and dried in an oven at 100 °C. Swelling power was then calculated as the ratio of the weight of the pellet to the initial starch sample weight. Solubility was calculated as the ratio of solids in the supernatant to the weight of the starch sample (dry basis).

2.3.3.3. Starch granules size distribution

The size distribution of the granules from soft wheat and bean starches was determined using a Mastersizer 3000 laser diffraction particle size analyzer equipped with a hydro HV dispersion system (Malvern Panalytical Ltd, Malvern, UK) and reported as the volume mean diameter $D_{4,3}$. The samples were suspended in water and stirred at 2000 rpm within the dispersion unit. The particles refractive and absorption indices were set at 1.53 and 0.1 respectively. All measurement were performed within the 0.1-10% obscuration range and with a 1.33 refractive index for water as the dispersant.

2.3.3.4. Scanning electron microscopy (SEM)

The morphology of the isolated starch granules was observed using a SU-70 Hitachi scanning electron microscope (Hitachi, Pleasanton, CA). The starch samples were adhered to a conductive carbon tape and dried in a vacuum oven at 3000 Pa at room temperature (21 °C). Subsequently, the samples were mounted onto specimen stubs and coated with a conductive gold layer using a sputter coater (Anatech, Hayward, CA) before imaging.

2.3.4. Characterization of pasta

2.3.4.1. Pasta preparation

The minimum water content for dough formation with wheat flour or bean flours as sole ingredient was evaluated and recorded as follows: 60% (w/w, db) for soft wheat, 40% for garbanzo and 55% navy bean flour, respectively. Wheat and two beans flours were hydrated with distilled water to the minimum water and then mixed for 10 min using a Kitchen Aid Mixer (St. Joseph, MI) equipped with the paddle attachment to develop a dough. The dough was passed through a pasta press extrusion plate held by the Kitchen Aid to obtain spaghetti. The pasta strands were dried at room temperature for 24 h before other measurements.

2.3.4.2. Cooking quality of pasta

The cooking quality of pasta was determined by following the AACCI 60-50 approved method. The optimal cooking time was established as the cooking time at which the central white core of a noodle strand disappears when squeezed between two pieces of glass.

To determine the pasta cooking loss, 10 grams of each sample were cooked to its optimum, and the cooking water was recovered and evaporated to dryness in a forced air oven at 100 °C for 12 hours. The solids were weighted, and cooking loss reported as the percentage of the original sample before cooking.

To determine the water uptake of pasta, 10 g of pasta were cooked to optimum, rinsed in cold distilled water and drained in paper towels before being weighted. The water uptake value was calculated as:

Water uptake (%, db) = $\left(\frac{\text{W(cooked pasta)}}{\text{W (dry pasta)}} - 1\right) \times 100$

2.3.4.3. Texture of pasta

To describe the texture of pasta, a texture profile analysis (TPA) was performed using a TA-XT2i Texture Analyzer (Stable Micro Systems Ltd., Surrey, UK). One pasta strand of approximately 7 cm length was placed under a flat-end cylindrical probe (d= 35 mm) and compressed to 70% of its original height. The test and post-test speeds were fixed at 1 mm/s. Hardness, cohesiveness, and chewiness were calculated from the resulting force versus time diagram. Ten measurements were taken and the average $\pm$ standard deviation reported.

2.3.5. Statistical analysis

This study was conducted as a completely randomized design. Each measurement was performed in triplicate and the results reported as the mean $\pm$ standard deviation unless otherwise specified. One-way ANOVA tests were carried out to find overall significant differences among the treatment groups and the Tukey's test to separate the means. The statistical analysis was performed using the RStudio Desktop 1.1.453 software (Boston, MA).

2.4. Results and discussion

2.4.1. Chemical composition of flours

The proximate compositions of the four bean flours were analyzed and compared to one commercial soft wheat sample (Table 2.1). As expected, wheat flour was especially different from the bean samples; it presented a significantly higher amount of starch (56.1%) and the lowest protein, lipids, and ash contents. Among the bean samples, small but significant differences ($p<0.05$) were found in starch, protein, lipid, and ash contents. Starch content ranged from 33.6 to 39.1%, where garbanzo and navy bean presented the highest and lowest contents respectively. These results are in agreement with the starch content in legumes within the 22-45% range as reported in previous works (Hoover & Ratnayake, 2002). The protein content presented less variation among the beans, great northern bean and navy bean did not significantly differ (~23.0%) but had a higher content than red kidney (21.8%) and garbanzo (20.3%). The lipids content of garbanzo was significantly higher than that from the other samples. Similar results have been reported, when comparing chickpea flours to those of *Phaseolus vulgaris* beans (Du et al., 2014). The ash content significantly differed among all the samples, with navy and garbanzo beans presenting the highest (4.85%) and lowest (3.23%), respectively.

2.4.2. Rheological properties of flours

It is known that the rheological behavior of doughs and the quality attributes of final goods are greatly influenced by the water absorption capacity of

flours, which varies among different flour sources (Yazar, Duvarci, Tavman, & Kokini, 2017). Hence, to simulate a scenario in which all the samples were under the same conditions, the rheological measurements were performed on flour suspensions, that is, in excess of water. A temperature sweep test, in which the samples were heated from 40 °C to 90 °C (Fig. 2.1A) and cooled back down to 25 °C (Fig. 2.1B) while applying a constant strain, was applied to the flour suspensions, to understand their rheological behavior under heat treatments such as cooking. In this work, soft wheat flour was included as a gluten-containing control.

As expected, the soft wheat presented the highest storage modulus (G') values, which was attributed to its significantly higher starch content and the presence of gluten in the flour (Table 2.1) (Salvador, Sanz, & Fiszman, 2006; Wu, Meng, Yang, Tao, & Xu, 2015). Garbanzo, which starch content was higher than the other beans, also presented the highest G' among the beans during the heating stage. The storage modulus of soft wheat sharply increased at about 60 °C due to the initial swelling of starch granules (Wu, Dai, Gan, Corke, & Zhu, 2016). On the other hand, bean flours showed a significant G' increase around 70-75°C. The delayed rise of G' in bean flours can be caused by the restricted swelling capacity of starches with higher amylose content (Hoover et al., 2010). It has been reported that starches with lower amylose content start their pasting at lower temperatures (Jane et al., 1999). This observation is in line with the amylose content as presented in Table 2.2 (18.2% for soft wheat while 26.4-

30.1% for beans). The Garbanzo bean starch with the lowest amylose content among beans (Table 2.2), presented an earlier increase of G' at 68 °C, while the significant increase of G' in the other three beans was found at 78 °C. The amylose contents in these three beans did not significantly differ (Table 2.2), supporting our hypothesis that starch amylose content was an important parameter in determining the rheological behavior of these bean flours. However, the rheological behavior of great northern, navy, and red kidney beans, with similar starch and amylose contents, was not identical, thus suggesting that other non-starch components such as protein might also affect the rheological behavior of bean flours.

Proteins are believed to form a matrix network which could entrap and protect the integrity of starch granules (Saleh, 2017). Their presence in flours has also been reported to reduce the water available to starch and to increase the temperatures at which starch granules start to absorb water (Baxter, Blanchard, & Zhao, 2014). These findings may help to explain the above observation that soft wheat and garbanzo flours with the lowest protein contents presented increases of G' at lower temperatures.

When the temperature was raised to 80 °C, the storage modulus of soft wheat decreased due to the melting of crystallinities, the disruption of the starch granular structure, and the leakage of amyloses from the granules (Fig. 2.1A)(Wu et al., 2016). On the other hand, G' continuously increased for all the bean flours in the whole period of heating, suggesting that the energy input was insufficient

to disrupt the structure of granules from bean starches. Heating the slurries to temperatures above the denaturation temperatures of bean proteins (80-90 °C) (Rui, Boye, Ribereau, Simpson, & Prasher 2011) could have denatured the proteins present in the flours, thus leading to the unfolding of their structures and subsequent gelation through hydrophobic interactions and disulfide bonds (Matos, Sanz, & Rosell, 2014), hence also contributing to increases of G'.

During the cooling stage (Fig. 2.1B), the interactions between the leached-out polymers from soft wheat granules brought the hot flour paste to an actual gel, as evidenced by a significant increase of G' from 475 to 2950 Pa (Yousefi & Razavi, 2015). As shown in Fig. 2.1A, the wheat starch granular structure was extensively disrupted at high temperature (85-90°C), which facilitated the lixiviation of amylose and amylopectin from the granules, thus yielding a hot paste rich in free polymers (Yousefi & Razavi, 2015). The higher G' of soft wheat flour (Fig. 2.1B) can be attributed to its higher starch content and the crosslinking of abundant free amylose and amylopectin molecules (Wu et al., 2015). The gluten portion may also contribute to the stronger network build-up. Among the beans, the garbanzo paste showed stronger elastic properties (higher G') than red kidney and great northern beans due to its higher starch content (Table 2.1) and lower starch gelatinization temperature. Nevertheless, navy bean, which showed lower G' values than garbanzo during heating, presented a rapid linear increase of G' upon cooling down and ended up with comparable G' values to garbanzo flour. It has been reported that the presence of native lipids in several

crops could interact with amylose and thus hinder the formation of junction zones among amylose molecules (Takahashi & Seib, 1988). Since garbanzo flour presented a higher content of lipids than navy bean (5.20% vs. 1.57%), these might have hindered the interactions among amylose molecules and decreased the elasticity of the garbanzo flour gels.

To further evaluate the viscoelasticity of the gels formed after the temperature sweep test, a dynamic frequency test was performed over the range of 0.1 to 25 Hz (Fig. 2.1C) (Wu et al., 2015; Wu et al., 2016). The low dependence of G' on frequency of all samples suggests high stability of these gels (Wu et al., 2016). Soft wheat showed higher G' than the bean samples, confirming a stronger gel network. Within the studied frequency range, navy bean and garbanzo bean showed a similar trend of G' values, which agrees on the results of the temperature sweep test (Fig. 2.1 B). Red kidney bean flour showed the lowest G' values in all the rheology measurements, indicating that this flour develops weak networks.

2.4.3. Physicochemical properties of starches

2.4.3.1. Swelling power and solubility

To better understand the different rheological behavior of these flours, their starch fractions were isolated and characterized. As shown in Table 2.2, soft wheat and garbanzo bean starch (with the lowest amylose content) presented significantly higher swelling capacity and solubility than the starches from great northern, navy, and red kidney bean. As postulated before, a higher amylose

content implies a closely packaging of amylose polymers within the amorphous regions of the starch granules (Hoover et al., 2010), which restricts the swelling of granules and requires high inputs of energy to start gelatinization (Tester and Morrison, 1990). These results agree with our observations on the rheology of flours slurries in that great northern, navy, and red kidney with higher amylose content presented higher gelatinization temperatures than soft wheat and garbanzo flours (Fig. 2.1A)

The correlations between amylose leaching, the swelling capacity of granules, and starch solubility have already been reported (Chung, Liu, Hoover, Warkentin, & Vandenberg, 2008; Tester and Morrison, 1990; Vandeputte, Derycke, Geeroms, & Delcour,2003). The leaching of amylose from granules is necessary to facilitate the swelling of amylopectin and the solubilization of starch granules. The higher swelling capacity soft wheat and garbanzo bean starches may indicate relatively weaker interactions between amylose-amylose and amylose-amylopectin molecules than in the other bean starches, thus facilitating the lixiviation of amylose molecules and increasing starch solubility (Chung, Liu, Donner, Hoover, Warkentin, & Vandenberg 2008).

2.4.3.2. Granule size and morphology

The morphology and size of starch granules are known to significantly affect the swelling and pasting properties of starches (Lindeboom, Chang, & Tyler, 2004). Here, the morphology and size of starch granules were characterized by SEM (Fig. 2.2) and laser diffraction analysis (Table 2.2),

respectively. All the four bean starch granules appeared to have oval to spherical shapes and lacked evidence of serious fissures, which agrees with previous reports (Hoover et al., 2010). Some striations were identified on the surface of granules, probably caused during milling before starch isolation (Baldwin, Adler, Davies, & Melia,1995). The laser diffraction results showed that soft wheat starch granules (12.9 µm) were significantly smaller than those from bean starches. As shown in Fig. 2.2, the granule size of soft wheat was variable with many small granules observed, which agrees with the laser diffraction measurement. Among the beans, garbanzo presented the smallest granule sizes (20.9 µm), whereas navy bean had the largest granules (28.0 µm) and great northern, and red kidney starch granules did not differ in their size (~26.0 µm). It has been reported that small granules tend to have less amylose content, lower pasting temperatures, and higher swelling capacity than large granules (Lindeboom, Chang, & Tyler, 2004; Singh & Kaur, 2004). Therefore, besides their lower amylose content, the smaller granule size also plays a crucial role in the higher swelling capacity of starch (Table 2.2) and lower gelatinization temperatures of soft wheat and garbanzo flours (Fig. 2.1A).

2.4.3.3. Pasting properties

In the present study, the pasting properties of starches and flours were studied using a Brabender Amylograpgh (Fig. 2.3). Three parameters including pasting temperature, peak viscosity, and final viscosity are reported in Table 2.3.

The pasting viscosity patterns of cereal starches such as soft wheat are classified as type B (Schoch & Maywald, 1968), that their large swelling capacity compromises the structure of granules and makes them susceptible to disruption under shear conditions after the peak viscosity is reached (Fig. 2.3). On the other hand, the pasting pattern of bean starches is classified as type C. These starches presented a more restricted swelling and their granular structure was resistant to mechanical fragmentation. Therefore, these beans did not show a clear peak viscosity or breakdowns. Similarly, the absence of peak viscosities and gradually increasing viscosities during the holding periods have been reported for different varieties of black bean, navy, pinto (*Phaseolus vulgaris* L.) and chickpea (C*icer arietinum* L.) starches (Hoover & Ratnayake, 2002).

As aforementioned, starch granules with smaller granule size and lower amylose require less energy to disrupt the polymer interactions within the granules, which facilitate granule swelling and starch pasting (Hoover et al., 2010). As expected, the garbanzo starch, with the smallest granules and lowest amylose content, showed the lowest pasting temperature (70.7°C) among the beans evaluated. Whereas great northern, navy, and red kidney starches with similar amylose content and granule size had similar pasting temperatures (78.4-79.5°C). Nevertheless, soft wheat starch, with the least amylose content, presented higher pasting temperature (73.9°C) than garbanzo starch. The pasting properties of starch might also be affected by amylopectin, especially for the starches with high amylopectin content such as soft wheat. For instance, the

long side-chains of wheat amylopectin interact through hydrogen bonds to form compact granule structure with high crystallinity which requires high inputs of energy to be disrupted, thus increasing the gelatinization temperature (Kohyama, Matsuki, Yasui, & Sasaki, 2004).

For most starches, the peak viscosity is mainly dictated by their swelling capacity (Schoch & Maywald, 1968). Nevertheless, in the present work, the peak viscosity did not perfectly correlate to the swelling capacity of the starches. The peak viscosity of garbanzo starch, with a higher swelling capacity, was slightly lower than that of great northern and navy beans (Table 2.3). Similarly, these three beans showed a higher peak viscosity than soft wheat, which had the highest swelling power (Table 2.2). It was found that the granule size of starch also affected the peak viscosity. Since larger granules would occupy more space within the measuring systems, they are expected to develop more viscous pastes (Singh, Singh, Isono, & Noda, 2010). Therefore, the larger granule sizes of starches from great northern and navy bean might explain why these starches showed higher peak viscosities than garbanzo starch and soft wheat starch.

During the cooling stage, the re-association of leached-out starch polymers and the interactions between intact remnant granules, dictate the extent of setback and final viscosity (Jane et al., 1999). The final viscosity of soft wheat starch was lower than that of garbanzo, great northern, and navy bean starches, which could be attributed to the higher degree of granule disruption during heating, and its lower amylose content, which limited the formation of

junction zones (Grant et al., 2001; Yousefi & Razavi, 2015). This observation is different from our rheological results measured on flours, in which soft wheat flour showed higher G' values than any bean flour after cooling (Fig. 2.1B). Nevertheless, the pasting properties of flours were also studied under same conditions (Table 2.3), and soft wheat flour showed the highest final viscosity among the five samples, which was in accordance with the rheological test. Thus, confirming that although soft wheat starch did not develop the strongest structures by itself, soft wheat flour formed the strongest gels due to its higher starch content and the presence of gluten.

Among the four beans, navy bean starch with the largest granules, showed the highest final viscosity, followed by garbanzo starch and great northern. Since free amylose molecules are mainly responsible for the formation of junction zones and increase the final viscosity (Grant et al., 2001). Hence, the low final viscosity values of kidney flour (191.7 mPa·s) and its starch (706.3 mPa·s) could be attributed to its limited ability to leach amylose, as evidenced by low swelling capacity and starch solubility. Compared to the isolated starches, all the flours presented lower peak viscosity and final viscosity values, and higher pasting temperatures (Table 2.3), this might be due to the presence of proteins and other non-starch components in flours, which may compete for water and protecting the integrity of granular structure (Li et al., 2016; Saleh, 2017). Therefore, more energy (higher temperature) is required for the gelatinization of starch in the flour. The SEM images (Fig. 2.2) show some residual proteins

deposited on the granule surfaces, which confirms that starch granules were surrounded by a protein matrix in the bean seed before isolation.

2.4.4. Cooking quality and textural properties of pasta

From the above experiments, garbanzo and navy bean flours showed better performance (stronger viscoelastic properties) than great northern and red kidney beans. Therefore, these two bean flours were selected to prepare pasta, and the cooking quality and textural properties of resultant pasta were compared with soft wheat as a gluten-containing control.

Table 2.4 shows the cooking quality and texture parameters of the pasta samples. Longer cooking was required for the soft wheat pasta to be cooked to its optimum, which might be due to a higher starch content and the presence of gluten. It is known that a strong protein network is formed by cross-linking of glutenin and gliadin which surrounds starch granules and restricts its swelling and gelatinization during cooking (Cubadda, Carcea, Marconi, & Trivisonno 2007). The pasta made from garbanzo flour presented the lowest optimum cooking time (5.5 mins), which is in accordance with our rheology and pasting observations that garbanzo flour and starch had lower pasting temperature than the navy bean sample (Fig. 2.1A, Table 2.3). The pasta made with navy bean flour showed a higher water uptake than garbanzo pasta (228 vs. 203%), which was inconsistent with the results of starch swelling power (Table 2.2). Therefore, other non-starch components of flour such as proteins might play an important role in the water uptake of cooked pasta.

Both bean samples showed higher cooking loss than soft wheat pasta. However, garbanzo bean pasta showed a lower value than navy bean sample (10.3 vs. 14.92%). The cooking loss for good quality wheat pasta should be lower than 12% (Hoseney, 1999). Considering the absence of gluten in the bean pasta, garbanzo bean might be acceptable and comparable to wheat and other commercial gluten-free pasta (Giuberti et al., 2015).

The texture parameters for cooked pasta are shown in Table 2.4. As expected, the two bean pasta showed lower hardness, cohesiveness, and chewiness than the soft wheat sample. The higher hardness, cohesiveness and chewiness values confirmed that the soft wheat was more elastic and had a stronger structure network than the beans after cooking, which was in accordance with our rheology measurement. For these three parameters, no differences were found between garbanzo and navy bean. As shown in Fig. 2.1B and Fig. 2.1C, the gel formed by navy bean flour could generate a structure as strong as garbanzo flour during the cooling stage of temperature sweep and dynamic frequency sweep. Thus, these two flours were expected to develop similar textural properties after cooking.

2.5. Conclusions

The physicochemical and rheological properties of four bean flours were studied and found to be significantly affected by starch characteristics such as amylose content and granule size. Our results showed that although soft wheat

starch did not develop the strongest structures by itself, soft wheat flour developed the strongest networks after a heat treatment due to its higher starch content and the presence of gluten. The starch amylose content and the granule sizes showed to be important parameters determining the pasting properties of flours, those flours which starches had the lowest amylose content and smallest granules, i.e., soft wheat and garbanzo flours, presented lower pasting temperatures. Additionally, due to the high amylose content of their starch (25.0%), the flours from the *Phaseolus vulgaris* and *Cicer arietinum* beans included in this study developed stable hot pastes as evidenced by continuous increasing storage modulus during the entire heating period of a temperature sweep test. Our rheology test results also showed that garbanzo and navy bean flours developed equally strong structures and the texture parameters of pasta made from these flours were not significantly different. Therefore, among the bean flours included in this study, garbanzo and navy beans could serve as ingredients to develop gluten-free pasta with better quality.

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Tables

Table 2.1. Proximate composition of soft wheat and bean flours. *

Flour	Starch	Protein	Lipids	Ash
Garbanzo	39.1 ± 0.3^{b}	20.3 ± 0.0^{c}	5.20 ± 0.0^{a}	3.23 ± 0.0^{d}
Great Northern	35.6 ± 0.0^{c}	23.2 ± 0.0^{a}	0.79 ± 0.0^{d}	3.95 ± 0.0^{c}
Red kidney	34.4 ± 0.0^{d}	21.8 ± 0.1^{b}	1.21 ± 0.0^{c}	4.07 ± 0.1^{b}
Navy	33.6 ± 0.7^{d}	23.1 ± 0.1^{a}	1.57 ± 0.0^{b}	4.85 ± 0.0^{a}
Soft wheat	56.1 ± 0.0^{a}	13.9 ± 0.0^{d}	0.72 ± 0.0^{d}	1.63 ± 0.0^{e}

*All the contents are expressed in g/100 g (db). Means in the same column with different superscripts indicate statistically significant differences (P>0.05).

Table 2.2. Physicochemical properties of starches from soft wheat and beans*.

Starch	Amylose content %	Swelling power (g/g)	Solubility %	Size distribution (µm)	Granule size (µm) D4,3
Garbanzo	26.4 ± 0.7^{b}	7.2 ± 0.1^{b}	4.0 ± 0.1^{b}	9.2 – 37.6	20.9 ± 0.6^{c}
Great Northern	29.5 ± 0.5^{a}	2.7 ± 0.1^{d}	1.0 ± 0.1^{c}	11.9 – 55.2	26.4 ± 0.4^{b}
Red kidney	29.6 ± 0.8^{a}	3.1 ± 0.0^{c}	1.5 ± 0.1^{c}	10.5 – 55.2	26.9 ± 0.4^{b}
Navy	30.1 ± 0.1^{a}	3.0 ± 0.0^{c}	1.0 ± 0.1^{c}	13.5 – 55.2	28.0 ± 0.3^{a}
Soft wheat	18.2 ± 1.0^{c}	9.6 ± 0.2^{a}	9.0 ± 0.3^{a}	2.6 – 27.6	12.9 ± 1.5^{d}

*Means in the same column with different superscripts indicate statistically significant differences (P>0.05).

Table 2.3. Pasting properties of soft wheat and common bean starches and flours. *

Sample	Peak viscosity (Viscosity at 95 °C, mPa*s)		Final viscosity (mPa*s)		Pasting temperature (°C)	
	Starch	Flour	Starch	Flour	Starch	Flour
Garbanzo	932.0 ± 13.2^{c}	295.0 ± 2.6^{b}	1369.7 ±9 .9^{b}	380.7 ± 3.2^{d}	70.7 ± 0.4^{c}	77.6 ± 0.0^{c}
Great Northern	986.0 ± 6.5^{b}	255.3 ± 8.4^{c}	1328.0 ± 2.6^{c}	568.0 ± 7.0^{b}	79.5 ± 0.1^{a}	81.0 ± 0.7^{b}
Red kidney	480.3 ± 4.5^{e}	70.7 ± 4.7^{e}	706.3 ± 2.3^{e}	191.7 ± 8.9^{e}	79.5 ± 0.3^{a}	86.8 ± 2.3^{a}
Navy	1180.0 ± 5.0^{a}	115.3 ± 2.9^{d}	1478.3 ± 4.7^{a}	410.7 ± 7.0^{c}	78.4 ± 0.3^{a}	87.3 ± 0.1^{a}
Soft Wheat	835.4 ± 2.5^{d}	451.3 ± 5.3^{a}	1266.0 ± 3.5^{d}	770.5 ± 7.3^{a}	73.9 ± 0.8^{b}	78.4 ± 0.3^{c}

*Means in the same column with different superscripts indicate statistically significant differences (P>0.05).

Table 2.4. Cooking and texture properties of pasta. *

Sample	Cooking time (min)	Water uptake (%, db)	Cooking loss (%, db)	Hardness (N)	Cohesiveness	Chewiness
Garbanzo	5.5 ± 0.7^{c}	203.0 ± 5.0^{b}	10.3 ± 0.1^{b}	10.6 ± 0.9^{b}	0.2 ± 0.0^{b}	1.7 ± 0.3^{b}
Navy	9.5 ± 0.27^{b}	228.0 ± 2.7^{a}	14.9 ± 0.5^{a}	10.5 ± 0.7^{b}	0.2 ± 0.0^{b}	1.5 ± 0.1^{b}
Soft wheat	10.5 ± 0.7^{a}	200.0 ± 1.9^{b}	6.0 ± 0.5^{c}	14.3 ± 2.1^{a}	0.3 ± 0.0^{a}	3.0 ± 0.6^{a}

*Means in the same column with different superscripts indicate statistically significant differences (P>0.05).

Figures

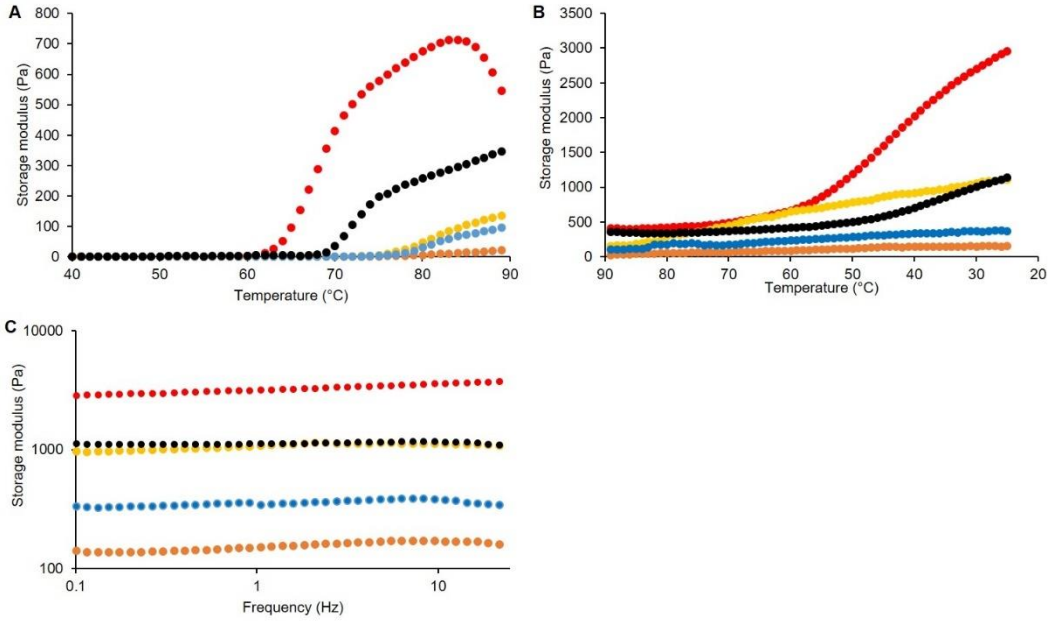

Figure 2.1. Viscoelastic behavior of bean and soft wheat flour suspensions (10% solids) as a function of (A) increasing temperature, (B) decreasing temperature, and (C) increasing frequency at 25 °C, respectively. Samples include garbanzo (black), great northern (blue), navy (yellow), red kidney (orange), and soft wheat (red).

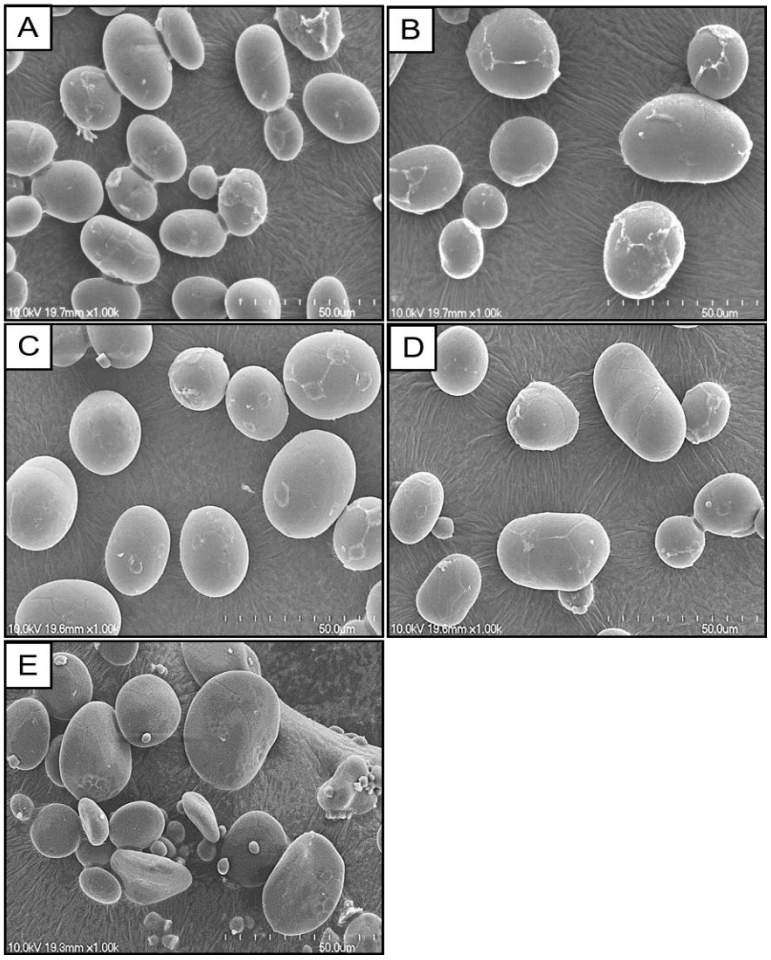

Figure 2.2. Scanning electron microscopy images of starch granules isolated from A) Garbanzo, B) Great northern C) Navy, and D) red kidney bean, E) soft wheat.

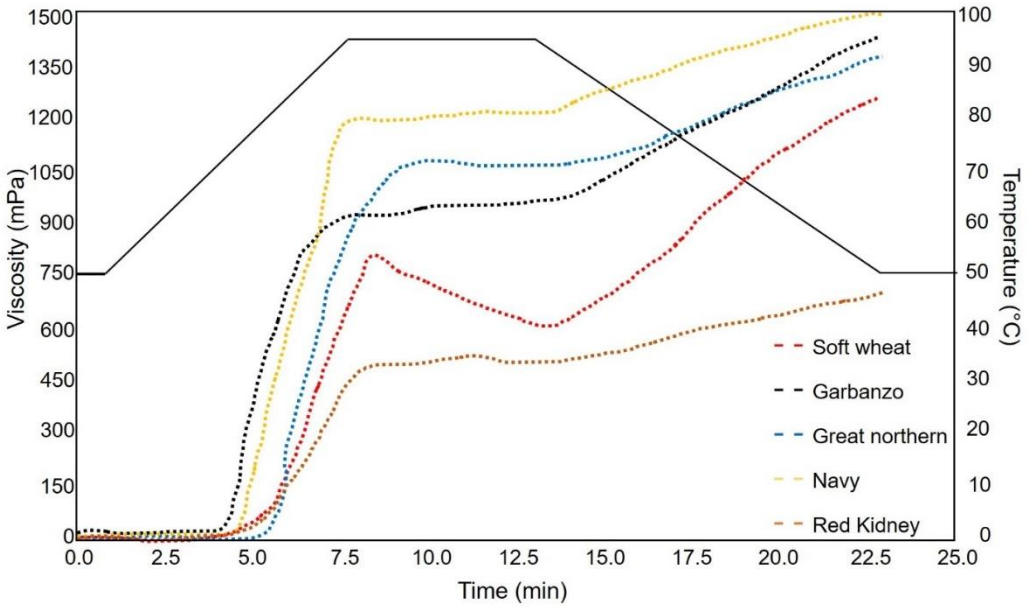

Figure 2.3. Pasting properties of soft wheat and bean starches (10%, db).

CHAPTER 3. EXPLORING THE STRUCTURE-FUNCTION RELATIONSHIP OF GREAT NORTHERN AND NAVY BEAN (PHASEOLUS VULGARIS L.) PROTEIN HYDROLYSATES: A COMPREHENSIVE STUDY ON THE EFFECT OF ENZYMATIC HYDROLYSIS

3.1. Abstract

The effect of enzymatic hydrolysis on the emulsifying properties of proteins from great northern and navy beans (*Phaseolus Vulgaris* L.) was studied and the causes of functionality improvements were comprehensively investigated. It was found that the exposure of the initial protein concentrates to the thermal hydrolysis conditions (no enzyme added) denatured their structures and significantly improved their emulsifying properties. Compared with initial protein concentrates, the alcalase and papain hydrolysates prepared at different enzyme-to-substrate ratios (E:S) showed improved interfacial and emulsifying properties. Nevertheless, only the use of alcalase at an 80:1000 E:S and papain at 1 and 5:1000 E:S produced stable emulsions with smaller droplet sizes than the heat-treated proteins from either source. It is concluded that proteins from common beans and their hydrolysates are promising alternative emulsifiers and that the inclusion of a heat-treated control is advisable to evaluate the effect of different hydrolysis levels on the functionality bean proteins.

3.2. Introduction

Dry beans (*Phaseolus vulgaris* L.), are the seeds of dicotyledonous plants belonging to the Leguminosae family, which are rich in proteins, minerals, and good sources of dietary fiber (Tharanathan & Mahadevamma, 2003). In addition to their high nutritional value, some health benefits such as reduction of the risk of cardiovascular diseases, obesity, and diabetes have also been reported on their consumption (Tharanathan & Mahadevamma, 2003). The United States is currently within the top ten largest producers of beans worldwide, even though most of the varieties from the genus *Phaseolus* are original from Central and South America (Bitocchi et al., 2012). However, these beans are usually consumed as cooked whole beans and its traditional cooking methods involve long soaking and cooking periods, which is seen as tedious and time-consuming by consumers, hence leading to the underutilization of these resources.

An alternative utilization of dry beans is the employment of their protein fraction as food ingredients. Protein isolates or concentrates, especially from animal sources, are commonly used as emulsifiers, foaming, and gelling agents by the food industry due to their excellent functional properties (Benjamin et al, 2014). Dry beans are reported to contain 20%-25% proteins with a relatively balanced amino acid composition and are considered as a cost-effective protein source, showing a potential to replace animal proteins (Du, Jiang, Yu, & Jane, 2014). However, when compared to animal proteins such as β-lactoglobulin,

legume proteins present limited foaming and emulsifying properties, as evidenced by higher interfacial tensions of oil/water interfaces, larger emulsion droplets, and lower zeta-potentials of emulsion droplets (Benjamin et al, 2014) which have limited their utilization in food applications.

The functional properties of proteins can be improved by controlled enzymatic hydrolysis. A large variety of enzymes including alcalase (Jamdar et al., 2010), chymosin (Barać et al., 2011), trypsin (Drago & González, 2000), and papain (Wani, Singh, Shanker, & Singh, 2015), have been utilized to produced hydrolysates from plant proteins with generally better solubility than the initial native proteins (parent proteins). (Drago & González, 2000; Yust et al., 2010). Nevertheless, the enzymatic hydrolysis of plant proteins does not always lead to functionality improvements (Avramenko, Low, & Nickerson, 2013; Jamdar et al, 2010). Therefore, the selection of an adequate enzyme is important when aiming to produce hydrolysates with improved functional properties.

The use of alcalase and papain to treat proteins from dry beans (*Phaseolus vulgaris* L.) have yield promising results. For instance, the emulsifying properties of black bean proteins have been improved by alcalase treatment (Evangelho et al., 2017) while the emulsifying capacity of kidney bean proteins was improved by hydrolysis with papain (Wani, Singh, Shanker, & Singh, 2015). However, the effect of alcalase and papain might vary depending on the bean market class taken as the protein source and on the degree to which the protein concentrates are hydrolyzed. Furthermore, in most of the works which

aim to improve the functional properties of proteins through hydrolysis, the causes of functional improvements are inadequately investigated to some extent. Since enzymatic hydrolysis is expected to yield small soluble peptides, and to expose hydrophobic and ionizable residues from the parent proteins, the characterization of size distribution, surface hydrophobicity, and interfacial properties of the obtained hydrolysates could provide a better insight of the reasons of functional improvements.

The enzymatic hydrolysis process can be divided into two main steps: i) the exposure of proteins to the optimum hydrolysis conditions (temperature and pH) for a certain time to reach the desired degree of hydrolysis, and ii) heating the hydrolysates to 85-100 °C to inactivate the enzymes and to stop the hydrolysis. Besides the proteolysis, the exposure of native proteins to the hydrolysis thermal and pH conditions and to the enzyme inactivation temperatures could also affect their functionality (Cui et al, 2014). Therefore, for a more comprehensive understanding of the effect of proteolysis (itself) on protein functionality, it is imperative to include a control sample which subjects the native proteins to all the hydrolysis conditions except to the exposure to the enzyme.

To the best of our knowledge, the effects of hydrolysis on the functionality of proteins concentrates from great northern and navy beans have not been studied before. Therefore, the first objective of this work was to characterize the structure-function relationship of great northern and navy bean protein hydrolysates as produced using alcalase or papain by relating the protein

solubility and emulsifying properties to changes in physicochemical properties such as surface hydrophobicity, interfacial properties, molecular size distribution, and protein net charge. The second objective was to comprehensively investigate the impact of enzymatic hydrolysis (including enzyme and exposure to hydrolysis conditions) on the functionality of great northern and navy bean proteins.

3.3. Materials and methods

3.3.1. Materials

Great northern and navy bean seeds were purchased from a local grocery store. Papain from papaya latex (≥10 BAEE units/mg) and alcalase (≥0.75 Anson units/mL) were obtained from Sigma-Aldrich (St. Louis, MO) and Millipore Sigma (Burlington, MA) respectively. All other chemicals such as sodium hydroxide, hydrochloric acid, sodium azide, and trinitrobenzenesulfonic acid were also purchased from Sigma-Aldrich.

3.3.2. Protein extraction

The bean protein concentrates were prepared according to the method of Rui et al., 2011 with modifications (Rui et al, 2011). Great northern and navy bean seeds were soaked in distilled water with 0.02% w/v sodium azide at 4°C overnight and manually dehulled. Subsequently, dehulled seeds were wet ground with distilled water in a 1:10 mass ratio, the resulting slurries were subjected to alkaline extraction (pH 9.5) for two hours, passed through a 75-µm-mesh, and centrifuged at 1600 g for 20 min to remove any insoluble material. The proteins

were precipitated by adjusting the supernatants to pH 4.5 and recovered by centrifugation (1600 g, 20 min). The pellet was washed twice with distilled water. Finally, the precipitated proteins were dispersed in distilled water, adjusted to pH 7.0, and spray dried in a B-290 mini spray-dryer (Buchi Labortechnik AG, Flawil, Switzerland). The inner flow rate and inlet temperature were set at 15 mL/min and 175 °C, respectively. The outlet temperature fluctuated between 75-80 °C. Both protein concentrates had a 75% protein content as determined by the Dumas combustion method using an FP-528 LECO apparatus (LECO Corp., St Joseph, MI) with a nitrogen conversion factor of 6.25.

3.3.3. Protein hydrolysis

Two and a half grams of protein (protein basis) were dissolved in 60 mL of distilled water (~4.1%) and adjusted to pH 7 and 55 °C before adding the enzymes. The protein samples were incubated with papain at enzyme-to-substrate w/w ratios (E:S) of 1, 5, and 20:1000 and with alcalase at ratios of 4, 8, 80:1000 for 90 minutes. The hydrolyses were carried out in a 150 mL jacketed beaker connected to a water bath to keep the temperature constant at 55 °C. The pH was controlled at 7.0 $\pm$ 0.1 using an autotitrator (Metrohm, Herisau, Switzerland) loaded with 1.0 M sodium hydroxide. After the hydrolysis, the enzymes were inactivated by placing the hydrolysates in a boiling water bath for 10 min. Then, the hydrolysates were placed in an ice bath immediately. In this study, two controls were included: the initial protein concentrates (control 1) and a protein sample which was subjected only to the effects of temperature and pH

for the same interval of time (control 2). After centrifugation at 10000 g, the obtained hydrolysates were dried in a Freezone 4.5 liter freeze dryer (Labconco, Kansas City, KS). The protein content of the controls and hydrolysates were determined to be 80-85% (Dumas method).

3.3.4. Degree of hydrolysis

The degree of hydrolysis (DH) which is defined as the number of peptide bonds cleaved during hydrolysis, was estimated by the trinitrobenzenesulfonic acid (TNBS) method proposed by Cayot and Tainturier, 1997 (Cayot & Tainturier, 1997). In brief, hydrolysates and controls were dissolved in 1.0% (w/v) sodium dodecyl sulfate (SDS) to obtain 5 mg/mL protein solutions. Aliquots (250µL) of these solutions were transferred to tubes containing 2.0 mL of phosphate buffer (0.2 M, pH 8.2). Subsequently, 2.0 mL of TNBS solution (0.1% w/v) were added, and the tubes were incubated in a 50 °C water bath for 60 min. Then, the reaction was stopped by the addition of 4 mL of 0.1 M HCl. The tubes were cooled down to room temperature before measuring the absorbance at 420 nm. The degree of hydrolysis was calculated with the following equation:

$$\text{Degree of hydrolysis (\%)} = \frac{(\mathrm{AN_2} - \mathrm{AN_1}) \times 100\%}{\mathrm{H_{tot}}}$$

Where AN_1 is the primary amino groups content (mmol leucine equivalents/ g protein) in the initial protein concentrate. AN_2 is the primary amino groups content of a sample after hydrolysis, and H_{Tot} is the primary amino groups content of the initial protein concentrate after total acid hydrolysis (6.0 N HCl, 100

°C, 24 h) (Avramenko, Low, & Nickerson, 2013). AN_1 and AN_2 were calculated using a standard curve of leucine.

3.3.5. Solubility

The protein solubility was measured according to Tang & Ma, 2009. The freeze-dried samples were dispersed in distilled water to obtain stock solutions with a protein concentration of 1mg/mL. The stock solutions were divided into five aliquots, and their pH was adjusted to 7, 6, 5, 4, and 3 respectively, followed by centrifugation at 7000 *g* for 10 min to remove any insoluble material. Finally, the protein concentration in the supernatant was determined by the Lowry method.

3.3.6. SDS-PAGE

The samples were mixed with 1× Laemmli sample buffer in the presence of β-mercaptoethanol (5%) and a total of 15 µg of each sample was loaded into a mini protean TGX Pre-cast (12%) gel (Bio-Rad Laboratories, Hercules, CA) for electrophoresis under a constant voltage of 200 V. After fixing and staining with Coomassie brilliant blue, the gel was distained and scanned using an Odyssey CLx imager (LI-COR, NE, USA). Apparent molecular weights were determined by using a broad range molecular weight standard marker (Bio-Rad Laboratories, catalog nr 161-0317).

3.3.7. Interfacial tension

The interfacial tension of soybean oil/water interfaces as stabilized by the protein samples was evaluated in accordance with Tamm, Herbst, Brodkorb, & Drusch, 2016 with some modifications. In brief, the protein samples were

dissolved in water at a protein concentration of 1 mg/mL. Then, the protein solutions were loaded to a DSA25 drop shape analyzer (Kruss, Hamburg, Germany) to create a pendant drop (~20 µL) in a cuvette filled with soybean oil. The interfacial tension (mN/m) was monitored for 5 minutes to evaluate changes in the oil/water interface due to protein absorption.

3.3.8. Protein hydrophobicity

Four mL of protein solution (0.01-0.1% w/v) were mixed with 20µL of 8mM ANS (8-anilinonaphthalene-1-sulfonic acid) and incubated in the dark for one hour. Subsequently, the fluorescence of the solution was measured with a LS55 fluorescence spectrophotometer (PerkinElmer, Waltham, MA) equipped with a quartz cell of 1.0 cm pathlength. The excitation wavelength was set at 350 nm, and the emission spectrum was recorded from 400 to 600 nm. The protein concentration was plotted against the fluorescence intensity at 470 nm, and the surface hydrophobicity was calculated as the slope of the linear regression of concentration versus intensity (S_0).

3.3.9. Preparation and characterization of emulsions

Oil/water emulsions were prepared by mixing 10.0 mL of aqueous protein solutions (1.0%, pH 7) with soybean oil to obtain a protein-to-oil mass ratio of 2:1, followed by homogenization using a T-25 ULTRA-TURRAX high-speed homogenizer (IKA, North Carolina, USA) at 14,000 rpm for 2.0 min. The emulsions were then stored at ambient temperature (18 °C) for one week. Sodium azide with a final concentration of 0.02% was added to the protein

solutions to prevent microbial growth during the storage of the emulsions. The average droplet size of emulsions was characterized during storage by dynamic light scattering (DLS) (Nano Zetasizer, Malvern Instruments, UK) with a fixed angle of 173° at 18 °C. The zeta-potential of freshly prepared emulsions was also determined using the Nano-ZS Zetasizer. To avoid multiple scattering effects, samples were diluted to 0.1% (w/v) protein concentration with distilled water before measurement.

3.3.10. Statistical analysis

All the measurements were carried out in triplicate. The data is presented as the average of at least three individual measurements ± the standard deviation. One-way ANOVA tests were carried out to find overall significant differences among the treatment groups and the Tukey's Honest Significant Difference post hoc test was used to separate the means. To evaluate the overall effect of the alcalase or papain hydrolysis, the average droplet sizes of emulsions prepared with hydrolysates produced at the different treatment levels were contrasted to the two controls included in this study (see supporting information). The statistical analysis was performed using the RStudio Desktop 1.1.453 (Boston, MA) free software.

3.4. Results

3.4.1. Enzymatic hydrolysis of great northern and navy bean proteins

The hydrolysis of the two protein concentrates by alcalase and papain at different E:S ratios was monitored by SDS-PAGE (Figures 3.1 and 3.2), and the

degree of hydrolysis (DH) was determined (Table 3.1). As shown in Figures 3.1A and 3.1B (lanes 2), the two protein concentrates showed many similar bands since they are varieties from the same species, *Phaseolus vulgaris* L. The storage proteins of *Phaseolus vulgaris* beans are mainly composed by the globulins 7S phaseolin and 11 S legumin. Phaseolin is a trimeric glycoprotein and each of its subunits has a molecular weight ranging between 43-53 kDa (Yin, Tang, Wen, & Yang, 2011). Whereas legumin is a hexamer composed by ~60 kDa subunits, and each subunit is composed by an acidic (~40kDa) and a basic (~30-20 kDa) polypeptide (Meng & Ma, 2001). The morphological subunits of both globulins are held together by non-covalent interactions. However, the acidic and basic polypeptides of the legumin subunits are linked by disulfide bonds (Staswick, Hermodson, & Nielsen, 1984).

Both samples presented a predominant band at 45 kDa (band V) which is ascribed to the subunits of the 7S phaseolin (43-53 kDa) (Rui et al, 2011). Under reducing conditions, legumin could be found as incompletely dissociated as legumin subunits (band IIIa, 60-70KDa) (López-Barrios, Antunes-Ricardo, & Gutiérrez-Uribe, 2016) and as its individual ~40 kDa acidic (band IIIb) and basic ~30-20 kDa subunits (Park, Kim, & Baik, 2010). An unidentified band IV at ~60 kDa has also been observed in a large variety of beans (Mojica, Chen, & de Mejía, 2015; Rui et al, 2011). Other predominant bean proteins such as linoleate 9S lipoxygenase (97 kDa, band II) (Mojica, Chen, & de Mejía, 2015), lectins including arcelin (López-Barrios, Antunes-Ricardo, & Gutiérrez-Uribe, 2016) and

phytohemagglutinins (band X, 29-35 kDa) (Makri & Doxastakis, 2006), inhibitors such as α-amylase inhibitors (band VII, 17-18 kDa) (Rui et al, 2011), and Bowman-Birk type proteinase inhibitors (band IX, 11 kDa) could also be observed in both beans.

Navy bean presented a strong band around ~30 kDa (band X), which might indicate a higher concentration of phytohemagglutinin. These lectins bind to glycoproteins on the membranes of erythrocytes or lymphocytes causing hemagglutination and are considered as antinutritional and allergenic compounds (Kumar et al., 2013). The hemagglutinating activity of phytohemagglutinins in navy bean has been reported to be significantly reduced by thermal treatments such as extrusion and steam cooking (Kelkar et al., 2012). It is possible that exposure of the initial protein concentrates to the enzyme inactivation thermal conditions caused the formation of insoluble aggregates (Mallikarjunan et al., 2014) from these lectins as evidenced by a faint ~30 kDa band after heat treatment (Figure 3.1B, lane 3).

The heat treatment to terminate the enzymatic hydrolysis caused the formation of water-soluble protein aggregates from great northern and navy bean proteins as evidenced by a darker band I around ~200 kDa (Figures 3.1A and 3.1B, lanes 3). Since incubating *Phaseolus vulgaris* L. protein concentrates above their denaturation temperature (82-91 °C) (Rui et al, 2011) disrupts the oligomeric structure of phaseolin, unfolds its subunits, and causes their aggregation (Tang & Ma, 2009), these aggregates could be mainly composed by

the 7S phaseolin. This hypothesis is supported by the slightly weaker band V in controls 2 as observed in Figures 3.1A and 3.1B (lanes 3), and by the fact that as band V was continuously hydrolyzed, the band I was gradually weakened in lanes 4-7. Similar results were obtained by Carrasco-Castilla et al., 2012 as the high molecular weight bands (~250 kDa) progressively faded as phaseolin was digested (Carrasco-Castilla et al., 2012). Additionally, a dark band on top of the gel is visible in controls 2, indicating that large aggregates formed and were unable to enter the stacking gel. The presence of this band has previously been observed after heating the albumin fractions of *Phaseolus vulgaris* L. bean proteins and has been attributed to the aggregation of lectins and trypsin inhibitors through disulfide bonds (Genovese & Lajolo, 1993). On the other hand, bands II and III, associated with lipoxygenase and legumin were not significantly affected by the processing conditions. The heat resistance of these proteins to similar mild heat treatments have also been reported for species belonging to the Fabaceae family (Park, Kim, & Baik, 2010).

After alcalase hydrolysis, the 7S phaseolin band in both samples remained in all hydrolysates (see Figures 3.1A and 3.1C, lanes 4-5) except the one treated at an E:S of 80:1000 (lanes 6). The major hydrolysis products for the alcalase treatment were in the 22-25 kDa range (Figures 3.1A and 3.1C). The central region of phaseolin was reported to be easily accessible to enzymes with different specificity (Deshpande & Nielsen, 1987). Therefore, a large variety of proteases could breakdown phaseolin into 22-25 kDa polypeptides which size is

approximately half of the former protein (Deshpande & Nilsen, 1987; Rui, Boye, Simpson, & Prasher, 2012). These resulting polypeptides were reported to be resistant to further hydrolysis unless their structure was altered by heat treatments before hydrolysis (Deshpande & Nilsen, 1987; Rui, Boye, Simpson, & Prasher, 2012).

The SDS-PAGE profiles of the two protein concentrates after papain hydrolysis are shown in Figure 3.2. The phaseolin band from great northern bean was completely hydrolyzed at an E:S ratio of 5:1000, while it was still present after hydrolysis in navy bean. It is remarkable that papain hydrolyzed most of the bean proteins to a larger extent than alcalase.

Lipoxygenase, legumin, and some lectins (band VI) were progressively digested by both enzymes (Figures 3.1 and 3.2). However, other polypeptides such as α-amylase inhibitors (bands VII), lectins (band VIII) and low molecular weight protease inhibitors (band IX) were resistant to hydrolysis by both enzymes, which may attribute to their relatively compact structure that limits the access of enzymes. The resistance of lectins in *Phaseolus vulgaris* beans towards hydrolysis by alcalase and papain was also identified in the work of Rui et al., 2012. Similar resistance to digestibility has been reported in *in-vitro* and *in-vivo* digestibility assays for the albumins of dry beans and other legumes (Park, Kim, & Baik, 2010; Salgado et al., 2003).

The degree of hydrolysis (DH) of the two bean protein hydrolysates as produced by alcalase and papain at different E:S ratios was determined. As

shown in Table 3.1, DH increased with the increasing E:S ratio for both enzymes. The highest degrees of hydrolysis were obtained by hydrolysis with alcalase at an E:S ratio of 80:1000, 56.9% and 46.0 % for great northern and navy bean respectively, which is consistent with the SDS-PAGE results that the bands, especially of phaseolin, faded away with the increasing E:S ratio. On the other hand, papain hydrolysates showed lower DH values but weaker bands than alcalase hydrolysates. Li et al., 2016 and Karamac, Kosinska-Cagnazzo, & Kulczyk, 2016 also reported that the electrophoretic patterns of soybean (*Glycine max* L.) and flaxseed (*Linum usitatissimum* L.) protein hydrolysates as produced by papain (DH < 10%) were mainly composed of low molecular size peptides (<10KDa), while alcalase hydrolysates with higher DH still presented some of the main storage protein bands in its electrophoretic profile. Similar observations have been reported on other protein hydrolysates produced by papain and alcalase (Mohan, Udechukwu, Rajendran, & Udenigwe, 2015). A few factors may be responsible for this incoherence: i) A larger amount of free amino acids may be present in the alcalase hydrolysates (Mohan, Udechukwu, Rajendran, & Udenigwe, 2015) thus increasing the number of primary amino groups available to react with TNBS (Cayot & Tainturier, 1997), hence, leading to a higher estimated DH value. ii) Random and spontaneous peptide degradation might have occurred during hydrolysis. For instance, intramolecular cyclization of the N-terminal of polypeptides and formation of diketopiperazines (Lancker, Adams, & Kimpe, 2011) could decrease the availability of primary amino groups, thus

leading to underestimations of the DH. Since short peptides exhibit less steric hindrance, these degradation mechanisms could have been favored in papain hydrolysates (Lancker, Adams, & Kimpe, 2011).

3.4.2. Effect of hydrolysis on the solubility of proteins

Protein solubility is considered one of the most important properties of proteins since many other functional properties such as emulsification and gelation are closely associated and rely on it. As shown in Figures 3.3A and 3.3C, both great northern and navy bean protein concentrates (controls 1) presented the typical U-shaped protein solubility profile with the lowest solubility observed at pH 5, which is close to the isoelectric point of these bean proteins (Rui et al, 2011). The thermal treatment itself significantly decreased the solubility of great northern bean proteins at pH 4 (Figure 3.3A, control 2), while a slightly reduced solubility of navy bean proteins was observed at pH 3 (Figure 3C, control 2). As previously discussed, water-soluble aggregates were formed in controls 2 by the heat treatment. It is possible that due to the lack of electrostatic repulsion at acidic pH condition, these aggregates were prompt to precipitate.

The enzymatic hydrolysis of both protein concentrates with either alcalase or papain significantly improved their solubility at the isoelectric point (Figure 3.3). These improvements occurred in a DH-dependent manner and are attributed to the reduction of protein molecular weight and to the exposure of ionizable residues previously buried within the protein structure, which creates additional repulsive forces among the peptides and prevents their aggregation

(Wouters et al, 2016). Nevertheless, at pH 3 or 4, lower solubility was observed in some alcalase and papain hydrolysates. This limited solubility of protein hydrolysates at acidic conditions have been reported before (Tsumura et al., 2005) and might be caused by excessive exposure of hydrophobic residues to the surfaces after hydrolysis and/or structural rearrangements because of pH changes (Alizadeh-Pasdar & Li-Chan, 2000), which promoted peptide-peptide interactions and their precipitation. Although great northern and navy bean proteins were equally soluble at pH 3 and 7, the alcalase hydrolysates of great northern bean showed slightly higher solubility than navy bean protein hydrolysates with similar DH value (29.0% vs. 28.4%) at pH 5 and 6, which may be ascribed to lower molecular sizes of these peptides. In contrast, navy bean hydrolysates as produced with papain showed better solubility than great northern hydrolysates.

The solubility of great northern or navy bean proteins at pH 7 was not affected by either enzymatic hydrolysis or heat treatment. Therefore, the following characterizations of hydrolysate samples and emulsion preparation were conducted at this pH condition to control any variance introduced by protein concentration.

3.4.3 Effect of hydrolysis on the emulsifying properties of proteins

Emulsification is one of the most important applications of food proteins. As indicated by McClements, 2014, when an excess of protein exists in an emulsion system, the droplet size distribution is relatively independent of the

protein concentration primarily depends on the energy input during homogenization. In this study, soybean oil was emulsified in a 2:1 protein-to-oil mass ratio, aiming to simulate the scenario in which excess protein existed in all emulsions. Subsequently, the droplet size distribution of emulsions was monitored for up to one week.

As shown in Figures 3.4A and 3.4C, heating the initial protein concentrates resulted in emulsions with significantly smaller droplet sizes (controls 2). For instance, the emulsions stabilized by the untreated great northern bean proteins had an average droplet size of 407.6 ± 16.9 nm, while those stabilized by the heat-treated proteins showed a droplet size of 307.5 ± 14.7 nm. Similarly, the droplet size of emulsions stabilized by navy bean protein decreased from 520.4 ± 12.8 nm to 382.0 ± 13.2 nm after the heat treatment. These results are in agreement with previous studies reporting that heat-treated soy proteins reduced the droplet size of emulsion (Cui et al, 2014).

To study the general effect of enzymatic hydrolysis on the emulsifying properties of bean proteins, the average droplet size of emulsions prepared with alcalase or papain hydrolysates at different treatment levels were compared to each of the two controls included in this study (Table 3.4). The hydrolysis with alcalase or papain significantly ($p < 0.05$) affected the droplet size of emulsions as compared to the controls. As expected, smaller differences were found between the different treatment levels and control 2 than these when compared with control 1 (Table 3.4). Additionally, although all the alcalase treatment levels were

significantly different from control 1 (both beans), those hydrolysates produced at E:S of 4:1000 and 8:1000 were not significantly different from control 2 over the storage period (Figures 3.5A and 3.5C). Nevertheless, when proteins were hydrolyzed at an E:S of 80:1000, the average droplet size of emulsions prepared with great northern (175.3 ± 6.0 nm) and navy bean (212.2 ± 8.3nm) hydrolysates was significantly reduced with respect to both controls. On the other hand, Figures 3.4B and 3.4D show that papain hydrolysis significantly decreased the droplet size of emulsions of both bean samples at all the treatment levels. However, in contrast to the alcalase treatment, the lower E:S ratios (1,5: 1000) further reduced the sizes of oil droplets when compared to the hydrolysate produced at 20:1000 E:S ratio.

The surface charge of these emulsions was also characterized by measuring the zeta-potential values of emulsions at pH 7 (Table 3.3). The control emulsions prepared by great northern bean and navy bean protein concentrates showed a negative zeta-potential of -34.0 mv, suggesting that repulsive electrostatic forces were contributed to the stabilization of emulsions (Wu, Eskin, & Cui, 2015). The heating treatment itself did not significantly affect the surface charge of emulsions (Table 3.3). The zeta potential value of both great northern and navy bean protein stabilized emulsions tended to decrease after alcalase hydrolysis at low E:S ratios, but the surface charge was increased when the protein concentrates were further hydrolyzed (E:S 80:1000). On the other hand, all the emulsions prepared with papain hydrolysates increased the surface

charge of oil droplets significantly as compared to the controls. As shown in Figure 3.2, papain hydrolysates were mainly composed by low-molecular-weight peptides, which may suggest that more ionizable groups were exposed to the surface of these peptides.

3.4.4. Effect of hydrolysis on the interfacial properties of proteins

3.4.4.1. Surface hydrophobicity

In the present study, the surface hydrophobicity of protein concentrates and their hydrolysates was determined with ANS as the fluorescence probe at neutral pH (Figure 3.5). The protein concentrates from navy bean presented a slightly higher hydrophobicity index than the proteins from great northern bean (73.5 ± 2.9 vs. 65.8 ± 4.1). The exposure of both protein concentrates to the hydrolysis conditions significantly increased their surface hydrophobicity (Figures 3.5A and 3.5C, controls 2). Similar results were obtained on heat-treated soybean proteins (*Glycine max* L.) (Cui et al, 2014) and other species from the genus Phaseolus (Tang, Sun, & Yin, 2009). Since most of the hydrophobic clusters are buried in the interior of the folded native proteins, the heating treatment was expected to partially denature the proteins and expose these hydrophobic residues to the surface. For a water-soluble protein source, more hydrophobic residues exposed to the surface would promote its interactions with the oil/water interface, thereby improving its emulsifying properties (Kato et al, 1981; Kato et al, 1985).

Enzymatic hydrolysis with papain (Figures 3.5B and 3.5D) gradually reduced the hydrophobicity of both bean protein sources at all E:S ratios, whereas the alcalase treatment decreased the hydrophobicity of great northern bean proteins (Figure 3.5A) and its effects on navy bean proteins (Figure 3.5B) depended on the extent of the hydrolysis (DH). Nevertheless, when compared with control 2, all alcalase and papain hydrolysates showed lower surface hydrophobicity values. The effect of enzymatic hydrolysis on the surface hydrophobicity of plant proteins have been wildly studied (Chen, Chen, Ren, & Zhao, 2011; Wu, Hettiarachchy, & Qi, 1998). It has been shown that at low DH, enzymatic hydrolysis indeed exposes the hydrophobic amino acid clusters to the peptide surfaces (Chen, Chen, Ren, & Zhao, 2011). However, further hydrolysis causes a decrease in hydrophobicity studied (Chen, Chen, Ren, & Zhao, 2011; Wu, Hettiarachchy, & Qi, 1998). This adverse effect of extensive hydrolysis may be attributed to two mechanisms: i) the enzymatic hydrolysis releases peptides with fewer hydrophobic sites to bind ANS (Wu, Hettiarachchy, & Qi, 1998), or ii) the ANS binding sites are reburied due to peptides aggregation through hydrophobic interactions (Chen, Chen, Ren, & Zhao, 2011). Additionally, besides the accessibility to the hydrophobic residues, the structural conformation of the proteins also affects the binding of ANS (Semisotnov et al., 1991). The fact that ANS shows greater affinity towards well-structured proteins with secondary structures rich in β-sheets than towards unstructured polypeptides (Semisotnov et al., 1991), might explain that the untreated and heat-treated proteins

presented higher hydrophobicity indices than the hydrolysates (Cui et al, 2014), which lost structure as the enzymatic hydrolysis progressed.

Since the emulsifying properties of the protein concentrates were significantly affected by enzymatic hydrolysis at all the treatment levels and did not directly depended on the surface hydrophobicity of the hydrolysates as measured by the ANS probe, other techniques were required here to elucidate the interfacial properties of these protein hydrolysates and the reasons of the improvement of their emulsifying properties.

3.4.4.2. Oil/water interfacial tension

To study the ability of the protein concentrates and hydrolysates from great northern and navy bean to stabilize oil/water interfaces, the interfacial tension of soybean oil/water interfaces was measured over a 5 minutes time interval. As shown in Figure 3.6, the interfacial tension was continuously reduced by all the samples due to their ability to diffuse and absorb onto the interface. However, in this short period, the interfacial tension did not reach the equilibrium. Therefore, to better understand the differences among the samples, the steady-state surface tension values (γ_∞) were estimated by plotting the interfacial tension versus $1/\sqrt{t}$ and taking the intercept of this linear regression as the tension at equilibrium ($t \rightarrow \infty$) (Wouters et al., 2017), as shown in Table 3.2.

After heat treatment, the γ_∞ of interfaces stabilized by both bean protein sources was slightly decreased, i.e., the γ_∞ of interfaces stabilized by great northern bean proteins went from 13.0 to 11.7 mN/m while γ_∞ decreased from

12.3 to 11.1 mN/m for navy bean proteins. The great northern and navy bean alcalase hydrolysates showed significantly lower interfacial tensions than both controls, indicating that these smaller peptides generated in the hydrolysis showed a greater affinity towards the interface. It is possible that the alcalase hydrolysis resulted in smaller peptides with higher diffusion rates and better structure flexibility which allowed them to undergo structural rearrangements at the interface (Wouters et al., 2017). Similarly, papain hydrolysates produced at the 1 and 5:1000 E:S ratios decrease the interfacial tension. However, hydrolysates produced at the 20:1000 E:S ratios increased the interfacial tension and were not significantly different to the control 2, indicating that extensive hydrolysis might yield short peptides with limited ability to absorb onto oil/water interfaces.

3.5. Discussion

To form stable emulsions, proteins must be able to absorb and undergo structural rearrangements at the surface of oil droplets during the homogenization of oil/water mixtures. Since the interactions between the proteins and the dispersed phase are mainly driven by hydrophobic forces, the surface hydrophobicity and structure flexibility of proteins are believed to be important parameters governing their emulsifying properties (Kato et al, 1985). Additionally, proteins must stabilize the emulsions by lowering the oil/water interfacial tension and generating electrostatic repulsive forces among the droplets to prevent the coalescence of emulsions (McClements, 2004).

In the present work, It was found that the heat-treated protein concentrates from either great northern or navy bean further reduced the interfacial tension of soybean oil/water interfaces when compared to the initial protein concentrates (Figure 3.6). This improvement was associated with a greater affinity of denatured proteins towards oil/water interfaces as a result of higher surface hydrophobicity (Cui et al, 2014), which was observed in our study (Figure 3.5, controls 2). According to the Stokes's law, most of the emulsion destabilization mechanisms rates are directly proportional to the droplet size (Sharma, Shukla, Misra, & Mishra, 2014). Smaller droplet size usually indicates a better emulsifying property of bean proteins. As shown in Figure 3.4, the heat-treated proteins from either great northern or navy bean produced smaller oil droplets than the initial protein concentrates, which perfectly correlated with the higher surface hydrophobicity and lower interfacial tensions as aforementioned. The emulsions prepared with the initial protein concentrates and the heat-treated proteins from both bean sources presented droplet charges larger than -30mv, which is considered the threshold to obtain colloidal stability (Wu, Eskin, & Cui, 2015). Additionally, the heat-treated proteins did not significantly affect the zeta-potential of emulsions. Therefore, it might be inferred that although the proteins were denatured, the number of ionizable amino acids exposed to the aqueous media upon absorption to the oil droplets was not changed.

Our results showed that all the alcalase hydrolysates from both bean protein sources had better emulsifying properties than the initial protein

concentrates (control 1). However, when compared with control 2, only those hydrolysates produced at the 80:1000 E:S ratio further decreased the droplet size of emulsions (Figure 3.4A and C, DH 56.9 and DH 46.0). The effect of alcalase hydrolysis treatment with lower DH values did not differ from that of heating the proteins. Therefore, in case such as improvement is desired, the hydrolysis treatment would not be needed when the heat treatment is enough to ensure such improvement.

In contrast to the heat-treated proteins, the droplet size of emulsions stabilized by some hydrolysates did not correlate either with the hydrophobicity nor the interfacial tension results, which indicates that the emulsifying properties of polypeptides do not only depend on their amphiphilic character but also on other factors such as their molecular size and structure flexibility (Kato et al, 1981; Kato et al, 1985; McClements, 2004). For instance, all navy bean hydrolysates produced with papain showed lower surface hydrophobicity and higher γ_{∞} than those of alcalase hydrolysates (Figure 3.5C and D, Table 3.2), while the emulsion stabilized by papain hydrolysates showed smaller droplet size. As shown in Figure 3.1, papain hydrolysates were mainly composed by short peptides, the lower molecular size of these hydrolysates might have favored their diffusion rates and flexibility during homogenization, then increasing their affinity, the ease of absorbtion, and structure arrangement at the interface, thus resulting in emulsions with smaller droplet sizes.

Figure 3.7 shows a schematic representation of the results obtained in this work. The exposure of the bean protein concentrates to the thermal conditions during enzymatic hydrolysis resulted in the unfolding of the protein structures and the exposure of hydrophobic amino acid clusters to the protein surfaces, which improved their affinity towards the oil/water interface but did not affect the surface charge of the oil droplets upon absorbtion. The alcalase treatment, especially that at 80:1000 E:S ratio, might have released unstructured and highly flexible polypeptides which efficiently lower the interfacial tension of oil droplets. Finally, the papain hydrolysis treatment resulted in the shortest polypeptides which might have favored their diffusion rates, exposure of hydrophobic and ionizable groups which improved their affinity towards the interfaces and generated the strongest repulsive forces among droplets (1,5:1000 E:S). Nevertheless, as previously reported (Betancur-Ancona et al, 2014; Yust et al, 2010), extensive hydrolysis of proteins (20:1000 E:S), released smaller peptides that form weak elastic films or inefficiently bind to the interface thus increasing the droplet sizes (Figure 3.4D) or creating unstable emulsions (Figure 3.4B). In this work, the fact that the emulsions stabilized by the 20:1000 E:S papain hydrolysates presented the highest zeta-potential might be explained by the presence of short peptides that did not efficiently bind to the oil droplets (Figure 3.7).

In this study, protein concentrates from great northern and navy beans were produced and treated with two enzymes including alcalase and papain at various E:S ratios. As a second control, the initial protein concentrates were

exposed to the thermal conditions of the hydrolysis process to study the effect of these conditions on the emulsifying properties. It was found that this heat treatment resulted in the denaturation of proteins and significantly affected the emulsifying properties. Therefore, the inclusion of this control is advisable for investigating the effect of hydrolysis more comprehensively. The alcalase hydrolysates prepared at the 80:1000 E:S resulted in emulsions with the smallest droplets and more negative zeta-potential values among the alcalase treatment levels, which features this treatment as a promising strategy to improve the emulsifying properties of both protein concentrates. On the other hand, papain hydrolysates with lower DH showed better emulsifying properties. Our study suggested that both bean protein sources are promising alternative emulsifiers and that appropriate enzymatic or thermal treatments can be applied to further improve their functional properties.

3.6. References

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Tables and Figures

Table 3.1. Degree of hydrolysis of great northern and navy bean protein hydrolysates as produced with alcalase or papain.[a]

Enzyme	E:S	Great northern (%)	Navy (%)
Alcalase	4:1000	21.4 ± 0.7[a]	13.1 ± 0.4[a]
	8:1000	29. 0 ± 0.8[b]	28.4 ± 0.3[b]
	80:1000	56.9 ± 1.2[c]	46.0 ± 4.2[c]
Papain	1:1000	4.6 ± 1.4[A]	7.8 ± 0.0[A]
	5:1000	13.1 ± 0.8[B]	16.6 ± 1.3[B]
	20:1000	32.1 ± 2.9[C]	28.6 ± 2.1[C]

[a] Different superscripts within each column and each protease treatment represent significant differences among means ($p<0.05$): alcalase, lower-case; papain, upper-case.

Table 3.2. Estimation of the interfacial tension at equilibrium (γ_∞) of soybean oil/water interfaces as stabilized by protein concentrates from great northern, navy bean controls, or their hydrolysates as produced with alcalase or papain[a].

Enzyme	E:S	Great northern (mN/m)	Navy (mN/m)
	Control 1	$13.0 \pm 0.1^{a, A}$	$12.3 \pm 0.1^{a, A}$
	Control 2	$11.7 \pm 0.2^{b, B}$	$11.2 \pm 0.1^{b, B}$
Alcalase	4:1000	9.7 ± 0.0^{c}	9.7 ± 0.1^{d}
	8:1000	9.1 ± 0.2^{e}	9.4 ± 0.1^{e}
	80:1000	9.5 ± 0.0^{d}	10.1 ± 0.0^{c}
Papain	1:1000	10.2 ± 0.1^{C}	9.9 ± 0.1^{D}
	5:1000	10.5 ± 0.1^{C}	10.2 ± 0.2^{C}
	20:1000	11.5 ± 0.1^{C}	11.4 ± 0.1^{B}

[a] Different superscripts within the same column represent significant differences among the means ($p<0.05$): alcalase, lower-case; papain, upper-case.

Table 3.3. Zeta potential of freshly prepared emulsions stabilized by great northern, navy bean protein concentrates and their hydrolysates.[a]

Enzyme	E:S	Great northern (mv)	Navy (mv)
	Control 1	-34.3 ± 0.8$^{a, B}$	-34.4 ± 0.9$^{b, C}$
	Control 2	-37.1 ± 1.5^{a,B}	-34.2 ± 3.9$^{b, C}$
Alcalase	4:1000	-29.1 ± 0.3^{b}	-33.6 ± 2.8^{b}
	8:1000	-27.5 ± 1.3^{b}	-35.1 ± 4.3^{b}
	80:1000	-33.8 ± 0.6^{a}	-43.9 ± 3.3^{a}
Papain	1:1000	-46.3 ± 1.4^{A}	-38.7 ± 2.1^{B}
	5:1000	-43.3 ± 2.3^{A}	-42.4 ± 3.4^{AB}
	20:1000	-46.5 ± 0.9^{A}	-45.8 ± 3.5^{A}

[a] Different superscripts within the same column represent significant differences among the means ($p<0.05$): alcalase, lower-case; papain, upper-case.

Table 3.4. Contrast test results for the comparison of the average droplet size (day 0) of emulsions prepared with great northern, navy bean proteins and the different levels of alcalase (A) or papain (P) hydrolysis treatments.

Protein source	Contrast	Diff. Estimate	Std. error	P values
Great northern bean	Control 1 vs. control 2	103.8	7.5	<0.05
	Control 1 vs. levels (A)	185.1	6.1	<0.05
	Control 2 vs. levels (A)	81.3	6.1	<0.05
	Control 1 vs. levels (P)	241.7	6.8	<0.05
	Control 2 vs. levels (P)	137.9	6.8	<0.05
Navy bean	Control 1 vs. control 2	126.6	15.7	<0.05
	Control 1 vs. levels (A)	217.7	12.8	<0.05
	Control 2 vs. levels (A)	91.1	12.8	<0.05
	Control 1 vs. levels (P)	308.9	12.3	<0.05
	Control 2 vs. levels (P)	182.4	12.3	<0.05

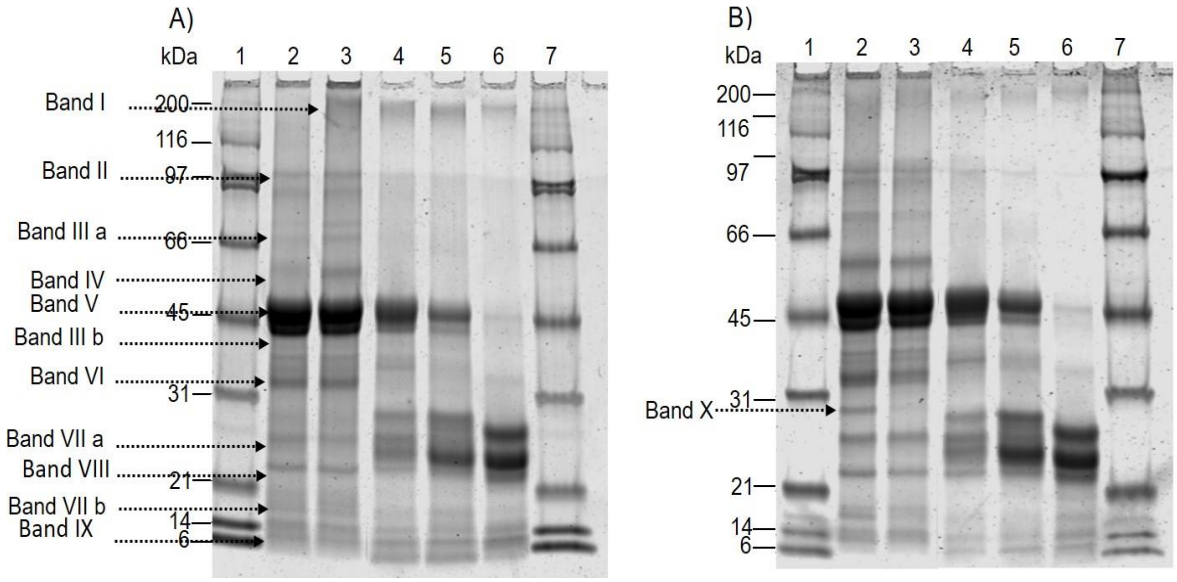

Figure 3.1. SDS-PAGE profile of A) great northern and B) navy bean protein concentrates and their hydrolysates as produced with alcalase at an E:S ratio of 4:1000, 8:1000 and 80:1000, respectively. Lanes 1 and 7 are molecular markers, while lanes 2 and 3 represent controls 1 and controls 2 in both gels. A: 4) DH 21.4%, 5) DH 29.0%, and 6) DH 56.9%. B: 4) DH 13.1%, 6) DH 28.4% and 7) DH 46.0%.

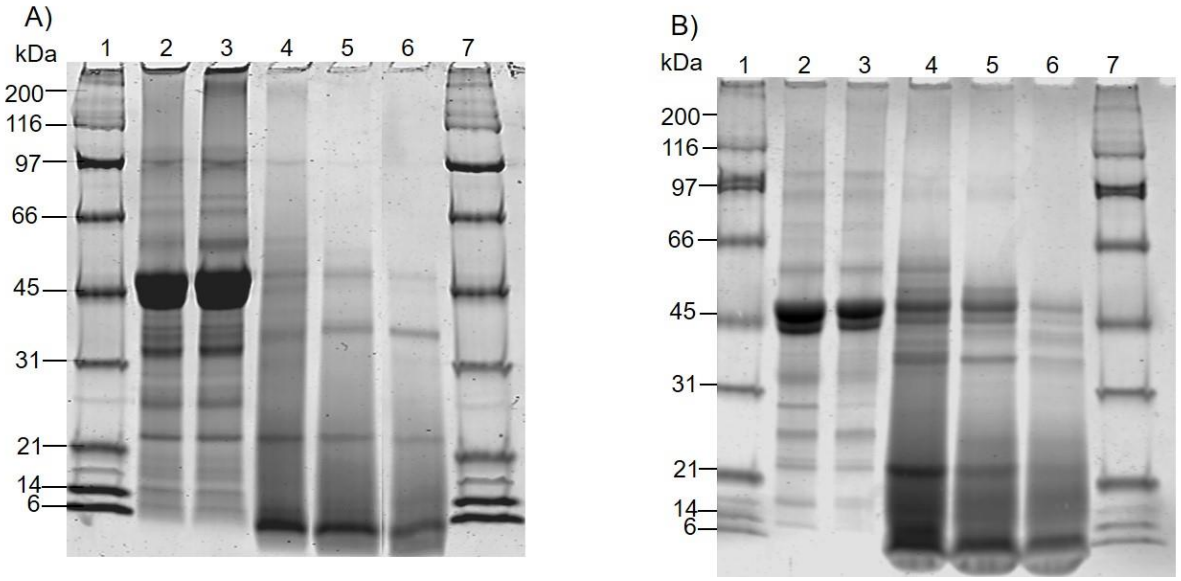

Figure 3.2. SDS-PAGE profile of A) great northern and B) navy bean protein concentrates and their hydrolysates as produced with papain at an E:S ratio of 1:1000, 5:1000 and 20:1000, respectively. Lanes 1 and 7 are molecular markers, while lines 2 and 3 represent controls 1 and controls 2 in both gels. A: 4) 4.6%, 5) DH 13.1%, and 6) DH 32.1%. B: 4) DH 7.9%, 5) 16.6%, and 6) 28.9%.

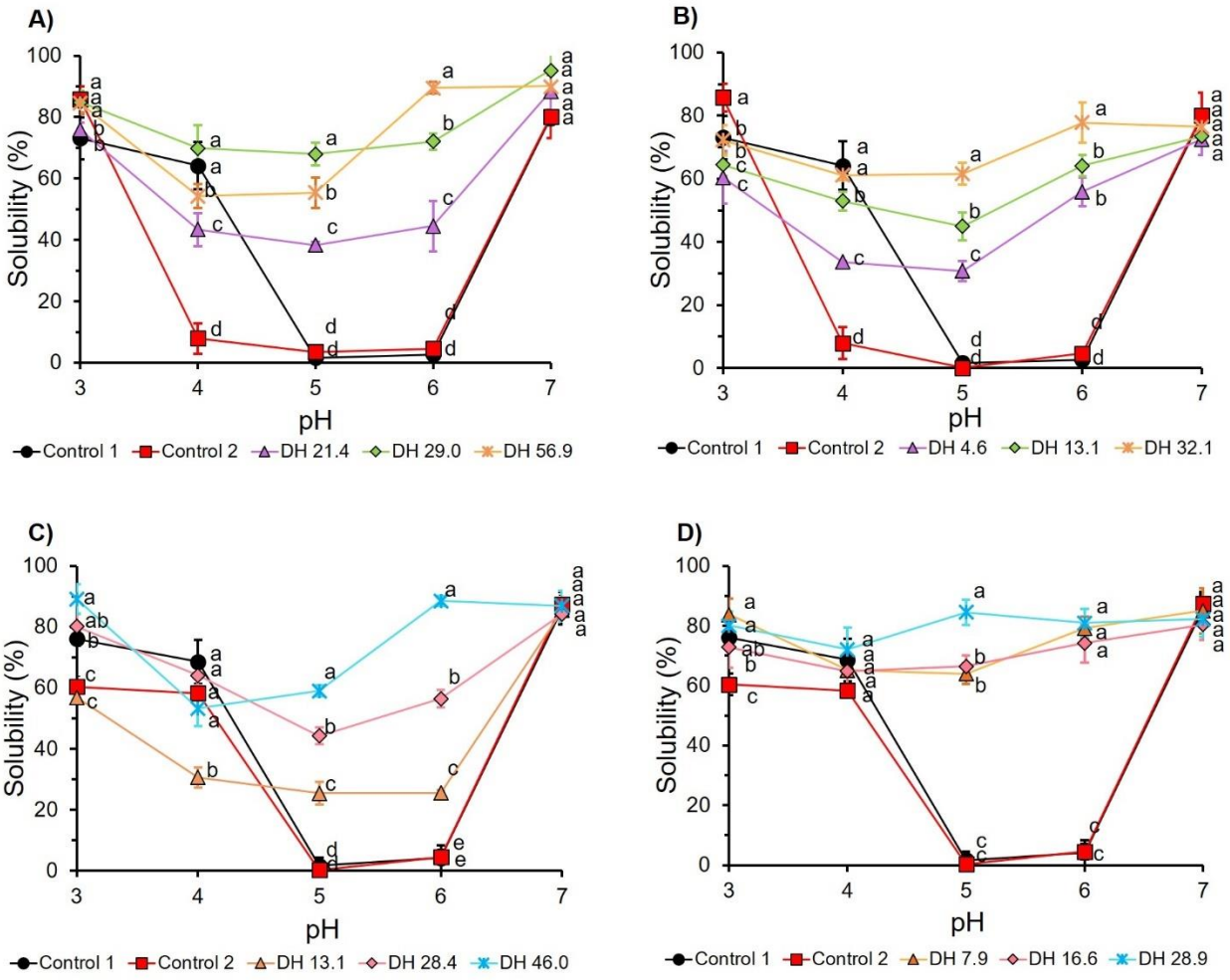

Figure 3.3. Protein solubility profile of great northern bean (top), navy bean proteins (bottom), and their hydrolysates as produced with alcalase (A, C) or papain (B, D).

Means within each pH condition accompanied by different superscripts presented significant differences ($p<0.05$).

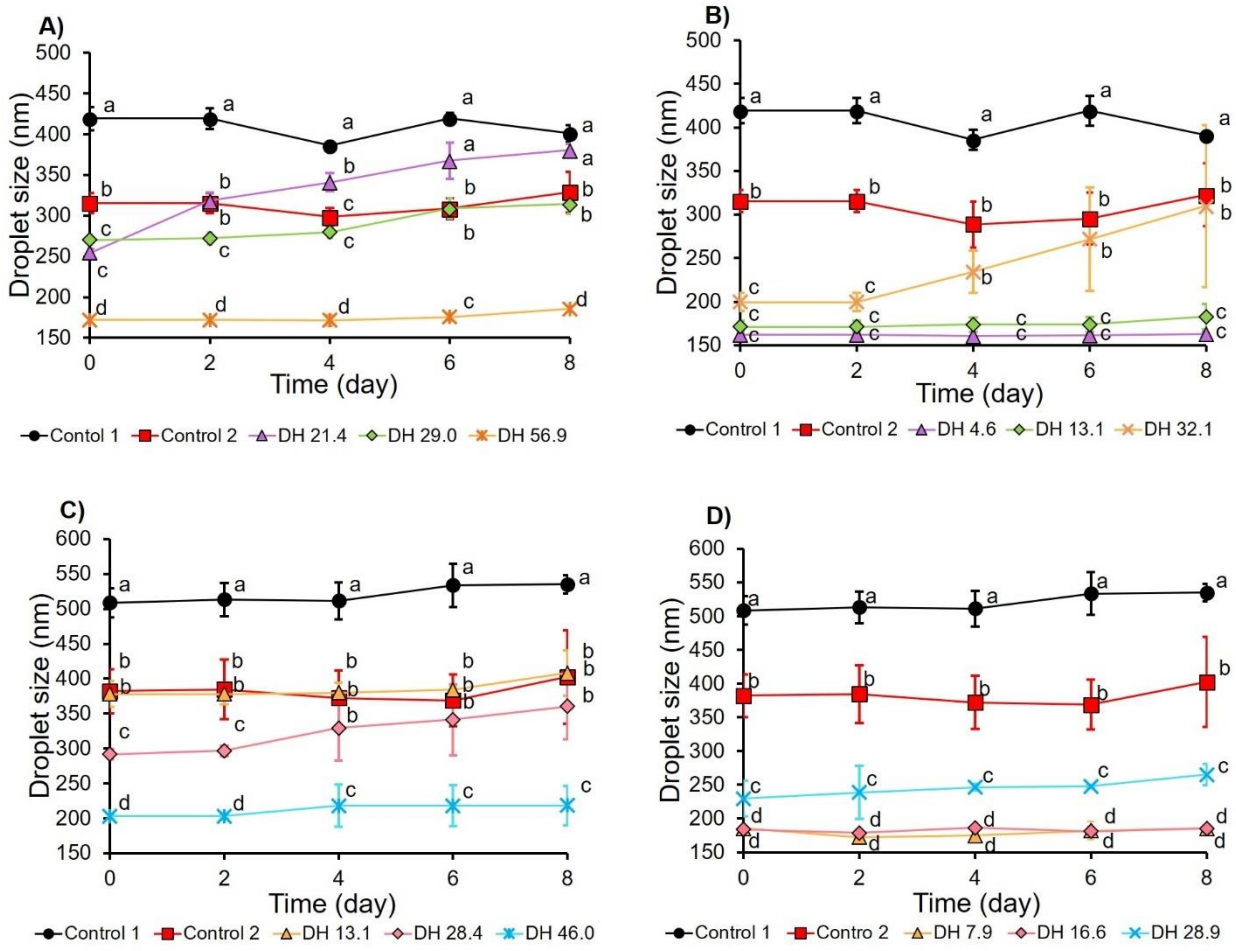

Figure 3.4. Average droplet size of emulsions stabilized by great northern bean (top), navy bean proteins(bottom) and their hydrolysates as produced with alcalase (A, C) or papain (B, D) during 8-day storage at 21 °C.

Means within each day accompanied by different superscripts presented significant differences ($p<0.05$).

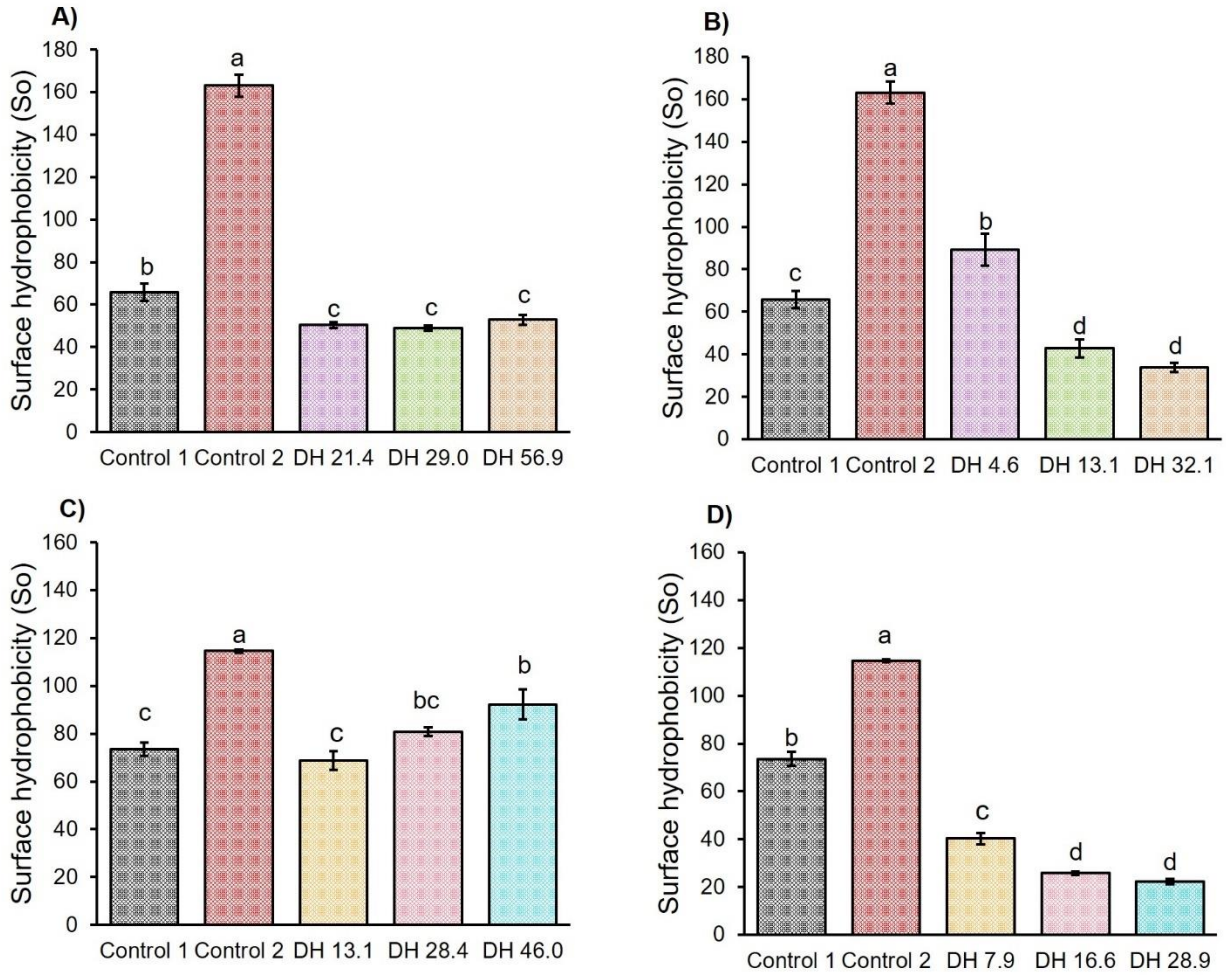

Figure 3.5. Surface hydrophobicity of great northern bean (top), navy bean proteins (bottom), and their hydrolysates as produced with alcalase (A, C) or papain (B, D).

Means accompanied by different superscripts presented significant differences ($p<0.05$).

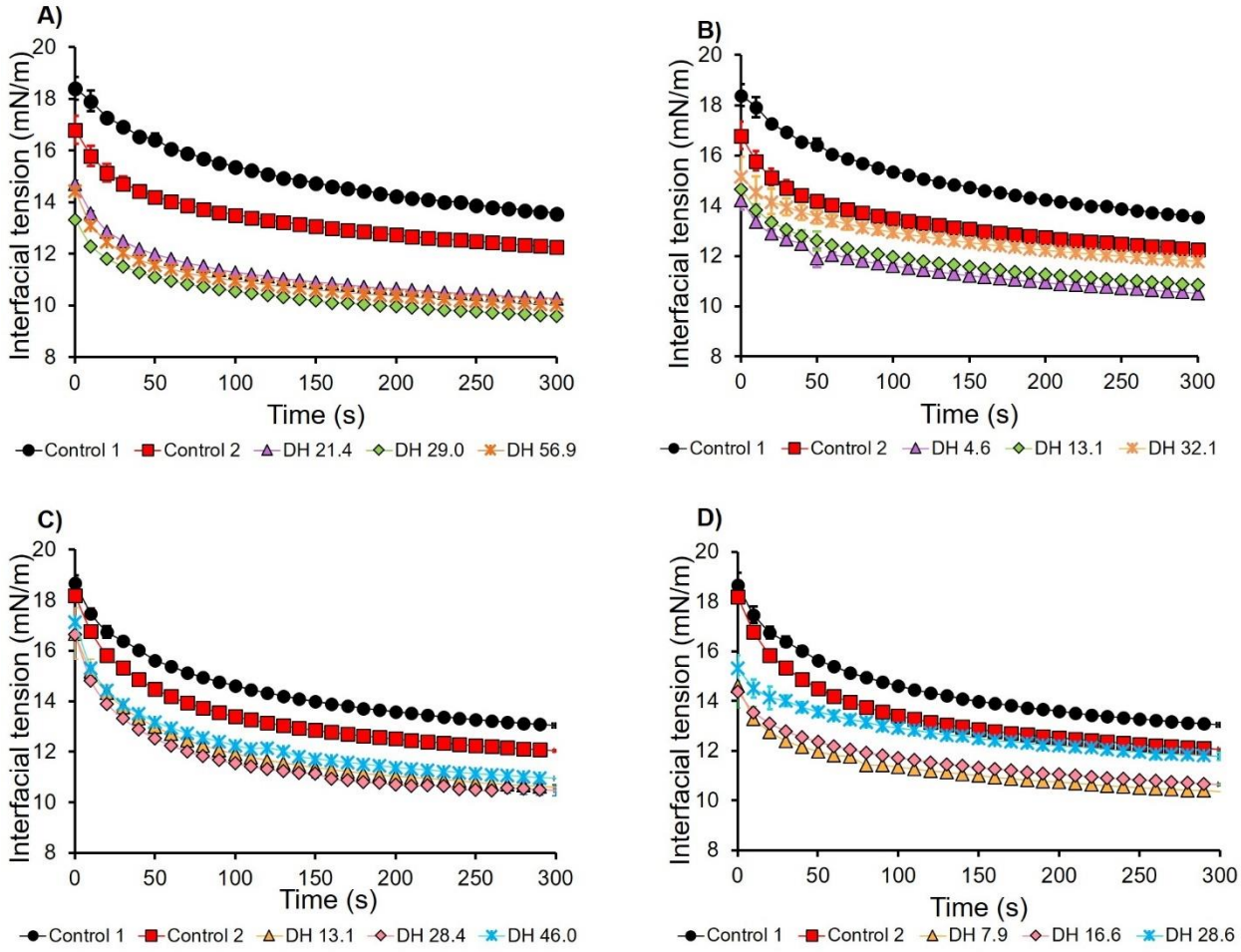

Figure 3.6. Interfacial tension of soybean oil/water interfaces as stabilized by great northern bean (top), navy bean proteins (bottom), and their hydrolysates as produced by alcalase (A, C) or papain (B, D) as a function of time.

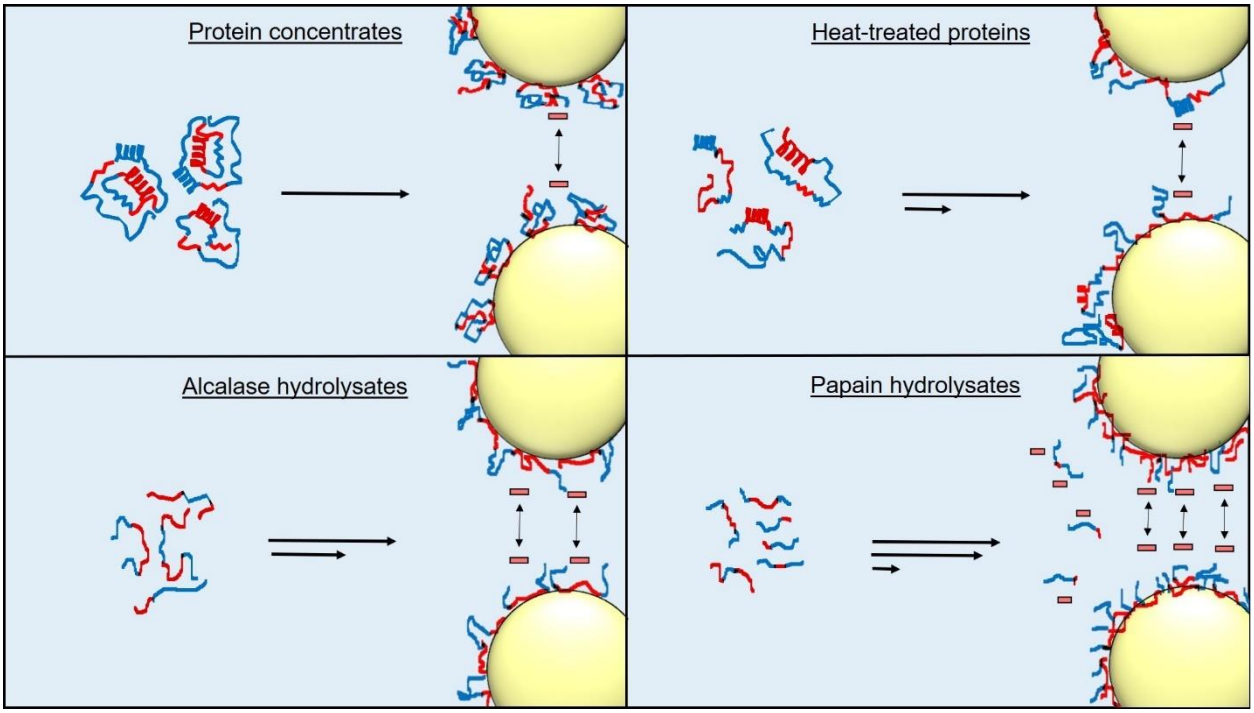

Figure 3.7. Schematic overview of the effect of heat treatment, alcalase, or papain hydrolysis on the emulsifying properties of protein concentrates from common beans.

4. OVERALL CONCLUSIONS

The first objective of this research was focused on studying the physicochemical properties of pulse flours and their starches, and the effect of these characteristics on the quality of resultant food products such as pasta, which is useful to promote the utilization of pulses by the food industry.

The relationship between the rheological behavior of four bean flours including great northern, navy, red kidney (*Phaseolus vulgaris L.)*, and garbanzo bean (*Cicer arietinum* L.) and the physicochemical characteristics of their starch fractions was explored. Although these flours presented similar starch (33-39%) and protein (20-23%) contents, their rheological behavior significantly differed from each other, indicating that they would behave differently during cooking.

It was found that among the characteristics of starch evaluated, the swelling and pasting properties of the starch fraction could be affected by the amylose content and the granule size. A higher amylose content was hypothesized to result in stronger intragranular interaction between starch polymers and to better protect the integrity of starch granules during heating. For this reason, the starches from *Phaseolus vulgaris* beans with higher amylose contents than garbanzo bean (30 vs. 26%) presented higher pasting temperatures and more restricted swelling behaviors.

Among the bean samples, the garbanzo and navy bean flours developed stronger gel-like structure than the other two beans, showing a potential to be

used as major ingredients in the development of food products which require strong and elastic structure (e.g., pasta). However, there is still a gap between wheat control and pasta made from 100% garbanzo or navy bean flours. Therefore, to efficiently utilize the flours from garbanzo and navy beans, the formulation of pasta must be optimized in case gluten-free pasta would be developed. However, the utilization of these flours should not be limited to the gluten-free market. Since garbanzo and navy flours develop strong elastic structures, these could also be utilized to enhance the nutritional quality of semolina pasta. It could be possible to incorporate garbanzo or navy bean flours into semolina flour to enhance the nutritional quality of semolina pasta while maintaining its texture quality.

Nevertheless, it is important to highlight that the findings and statements made in this work are limited to the four specific bean flours included in the study and that the population of inference should not be extended to the level of a whole species, i.e., all varieties in the species *Phaseolus vulgaris* L.

The second objective of this research was to study the effect of enzymatic hydrolysis on the functionality of great northern and navy bean proteins using alcalase or papain. Our results showed that the enzymatic hydrolysis at the enzyme-to-substrate ratios of 80:1000 for alcalase and 1:1000 and 5:1000 for papain, respectively, significantly improved the emulsifying properties of these protein concentrates. These improvements may be due to the reduction of the molecular size of proteins, the exposure of more ionizable amino acids to the

peptide surface, and a more flexible structure of hydrolysates which effectively reduced the interfacial tension between water and oil droplets. Our study suggested that both bean protein sources are promising alternative emulsifiers.

Although the emulsifying properties of these hydrolysates were only studied at pH 7, our results showed that the solubility of these bean proteins was improved at their isoelectric point. The improvement of their emulsifying and other properties such as foaming capacity at other pH condition could be expected. Further research on the comparison of the functionality of these bean protein hydrolysates with commercial protein ingredients, i.e., whey or soybean protein isolates, is recommended.