STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF RED KIDNEY BEAN (*PHASEOLUS VULGARIS*) PROTEINS AND ENZYMATIC PROTEIN HYDROLYSATES

By

Sule Mundi

A Thesis

Submitted to the Faculty of Graduate Studies of

University of Manitoba

In Partial Fulfilment of the Requirements for the Degree of

Doctor of Philosophy

Food and Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba

Canada

Abstract

Kidney bean proteins and peptides can be developed to serve as an important ingredient for the formulation of high quality foods or therapeutic products that may positively impact on body function and human health. The main goal of this thesis was to determine the *in vitro* structural and functional characteristics of major proteins and enzymatic protein hydrolysate of red kidney bean (*Phaseolus vulgaris*). Selective aammonium sulfate precipitation of the kidney bean proteins yielded 88% globulin and 7% albumin. The globulin and albumin are glycoproteins that contained ~4% and 45% carbohydrate contents, respectively. Physicochemical and functional characteristics of the globulin fraction, such as, gelation concentration, foam stability, emulsion capacity, and emulsion stability were superior to those of albumin. Reducing SDS-PAGE revealed vicilin with molecular weight of ~45 kDa as the major globulin in kidney beans. Circular dichroism spectroscopy of the purified vicilin showed reductions in α-helix, and β-pleated sheet conformations upon addition of NaCl or changes in pH. Likewise, the tertiary structures as observed from the near-UV CD spectra were also changed by shifts in pH conditions and NaCl addition. Far UV-CD showed increased β-sheet content up till 60^{o}C from room temperature, but a steady loss in the tertiary structure as temperature was further increased; however, β-sheet structure was still detectable at 80^{o}C. Differential scanning calorimetry thermograms showed a prominent endothermic peak with denaturation temperature at around 90^{o}C, attributed to thermal denaturation of vicilin. Alcalase hydrolysis of kidney bean globulin produced multifunctional peptides that showed potential antihypertensive properties because of the *in vitro* inhibition of activities of renin and angiotensin I converting enzyme as well as the antioxidant properties. The <1

and 5-10 kDa peptide fractions exhibited highest ($p<0.05$) renin inhibition and the ability to scavenge 2, 2-Diphenyl-1-picrylhydrazyl free radical, inhibit peroxidation of linoleic acid and reduce Fe^{3+} to Fe^{2+}. Based on this study, incorporation of kidney bean globulin as an ingredient may be useful for the manufacture of high quality food products. Likewise, the kidney bean protein hydrolysates, especially the <1 kDa fraction represent a potential source of bioactive peptides for the formulation of functional foods and nutraceuticals.

Acknowledgement

A number of good people graciously journeyed with me through the good and challenging times that I faced in my pursuit of this PhD degree, and I am indebted to open a page of very sincere gratitude to them.

I will like to begin in the name of God, the creator of universe, Most Gracious, Most Merciful who gave me the strength and provided the health for me to carry on with the requirements for the program. I am heartily thankful to my supervisor, Dr Rotimi Aluko, whose supervision, advice, encouragement, support and guidance from the initial to the final level enabled me to develop an understanding of the subject. An opportunity for a PhD under Dr Aluko's supervision taught me beyond an academic degree. I simply could not wish for a more understanding supervisor. His truly inspiring management strategies in addition to exceptional ideas and passions in science have enriched my growth as a student, a researcher, scientist and a manager.

I gratefully acknowledge and appreciate the efforts of Dr Michael Eskin and Dr Prashen Chelikani, members of my supervisory committee, whose useful guidance and contributions help shaped my thesis. My gratitude goes out to the Dean of Human Ecology, the Head of the Department of Human Nutritional Sciences as well as other members of the Human Nutritional Sciences and Food Science academic staff who made individual efforts to guide and support me at different times in the course of my program.

I was extraordinarily fortunate and feel very greatly honored to be a recipient of the Islamic Development Bank (IDB) Merit Scholarship for high technology. I therefore, wish to convey very special acknowledgement for IDB's generosity in providing the

excellent funding opportunity. IDB Merit scholarship solely paid my tuition and my living expenses for the three years with an exceptional privilege of extension. I couldn't otherwise have carried out my research without this opportunity.

Where would I be without my family? My parents deserve special mention for their inseparable support and prayers. My Father may his gentle soul rest in peace in heaven. He is no more alive now, but in the first place he was the person who put the fundamentals of my learning character, showing me the joy of intellectual pursuit ever since I was a child. You will always be a part of al1 my successes. My Mother is the one who sincerely raised me with her caring and gently love and her prayers still smoothen my path till today.

Words fail me to express my appreciation to my wife Aisha who doubles as my mother in her understanding, dedication, love and persistent confidence. I owe her a lot for never letting her intelligence, passions, and ambitions collide with mine. She has been an endless pillar of strength. Your love and support continues to reveal the meaning of marriage. My children, Fauziya, Farida, Rahmat and Mubarak have loved me without reservation, even at their very young age. They understand the value of support and prayers, and they have given me a lot of support and prayers. Without your love, little would be possible.

Finally, collective and individual acknowledgments are also owed to my past and present colleagues in Dr Aluko's laboratory, with whom I have worked, and whose presence were perpetually refreshing, helpful, and memorable. Many thanks go to them for giving me such a pleasant time when working together.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

1.0. INTRODUCTION AND LITERATURE REVIEW

1.1. General Introduction.

Legumes are of *Leguminosae* plant family, and constitute one of the world's most important groups of plants, second only to cereals in providing food crops for world agriculture (Duranti, 2006). In this sense, they represent an important source of dietary protein for large segment of the human population and domestic animals. Pulses as used by the Food and Agricultural Organization (FAO), including French, navy, pinto, field, haricot, China, marrow frijoles, snap, string, wax, black, white and kidney bean are the most widely produced and consumed food legume in Asia, South America, Latin America and Africa where they serve as good sources of protein with good nutritional and functional properties (Boye, Zare, & Pletch, 2010; Sathe, 2002). This is particularly true in those countries in which the consumption of animal protein is limited by scarcity, high cost or self-imposed because of religious or cultural habits (Boye et al., 2010). As the world population grows and the demand for reducing the risks related to consumption of animal food products increases, there is a corresponding growth for plant protein production, especially in the developed countries. For instance, FAO figures for bean production, the global dry bean production in 2008 was 20.39 million metric tons (MT), reflecting an increase of 48.74% over the 1980 production (Siddiq et al., 2011). The yield during this period increased from 0.54 MT/hectare in 1980 to 0.73 MT/hectare in 2008 (Siddiq et al., 2011). Canadian production of pulses has also shown an upward trend from 3.7 million tonnes in 2006 to 5.2 million tonnes in 2009 (Agriculture and Agri-food Canada, 2009). Canada exports 90% of its production mainly to the USA, the UK and Italy, usually destined for the food-processing sector (Agriculture and Agri-food Canada,

2009). Canadian export accounts for 35–40% of global pulse trade and therefore, developing new market opportunities and addressing barriers to consumption and trade are of paramount importance (Siddiq et al., 2011). Of the *Phaseolus* legumes, kidney bean is the most widely consumed legume in the world (Nielsen, 1991). In several countries, kidney beans are a staple food and serve as necessary supplement to other protein sources where protein energy malnutrition is prevalent (van Heerden & Schönfeldt, 2004). Kidney beans are processed as canned in brine, meat-based chili products, or acidified bean salad pack. They can be used in a variety of dishes including soups, salads, sandwiches, and dips (Osorio-Díaz et al., 2003) where they serve as a good source of vitamins (thiamine, riboflavin, niacin, vitamin B6, and folic acid), minerals (Ca, Fe, Cu, Zn, P, K, and Mg), and polyunsaturated fatty acids (PUFA), especially linoleic and linolenic acids (Augustin & Klein, 1989; Kutoš, Golob, Kač, & Plestenjak, 2003). Kidney beans are also rich in non-nutrient components, such as phenolics, and antioxidant compounds (Amarowicz & Pegg, 2008; Xu & Chang, 2008).

In addition to contributions to human nutrition, legume seeds have been unequivocally proven to represent key functional foods because they contain a number of compounds with potential beneficial health effects. Recent research studies suggest that consumption of dry beans in general may have potential health benefits including reduced risk of cardiovascular disease, cancer, diabetes, osteoporosis, hypertension, gastrointestinal disorders, adrenal disease and reduction of low density lipoprotein cholesterol (Hu, 2003; Jacobs & Gallaher, 2004; Tharanathan & Mahadevamma, 2003). In particular, an antidiabetic and antiobesity role for an α-amylase inhibitor from kidney bean (*P. vulgaris* L.) has been claimed (McCarty, 2005; Obiro, Zhang, & Jiang, 2008). Other biological

activities such as antitumor, antihistamine, immunomodulatory, antifungal, anti-human immunodeficiency virus (HIV) and anti-insect activities have also been reported for kidney bean proteins (Eskin & Tamir, 2006). Consequently, there is growing awareness in the potential of including kidney beans in the development of novel food products such as functional foods and nutraceuticals. One major advantage of using the kidney bean proteins would be their additional functionalities. Studies of the functional properties and *in vitro* protein digestibility of flours prepared from *Phaseolus* legumes, including kidney beans showed higher or in some cases similar gelation capacity, emulsifying activities and foam stability when compared to soybean flour (Tang, Sun, Yin, & Ma, 2008). Functional properties, such as emulsification, foaming or gelation can influence to a large extent the protein suitability as an ingredient in various food applications. Kidney bean protein concentrates and isolates could, therefore, be suggested as a potential supplement in several number of food applications offering opportunities for novel product development. However, despite the various nutritional and biological benefits, there are some undesirable characteristics that limit increased kidney beans consumption (Boniglia et al., 2008; Jourdan, Norea, & Brandelli, 2007; Oomah, Blanchard, & Balasubramanian, 2008). Some of these concerns include bioavailability and digestibility of the bean; anti-nutritional factors such as enzyme inhibitors and lectins, and raffinose-containing saccharides (Duranti, 2006). However if beans are well processed the side effects are reduced or even completely eliminated, and may contribute to food functionality.

In recent years many biological peptides which are inactive within parent proteins of various food, have been liberated during enzymatic digestion or food processing to exhibit health benefits, such as immune defense, uptake of nutrients, opioid activity,

antihypertensive activity, antioxidant activity, antibacterial activity, mineral-binding activity and enhancement of intestinal activity. In particular, some of the bioactive peptides found in hydrolysates from food proteins digested with different proteases under different hydrolysis conditions have been associated with the renin–angiotensin system, which regulates peripheral blood pressure via conversion of angiotensin I to angiotensin II (Ajibola, Eleyinmi, & Aluko, 2011; Medina-Godoy et al., 2012; Ruiz-Ruiz, Dávila-Ortíz, Chel-Guerrero, & Betancur-Ancona, 2012). Inhibition of angiotensin-I-converting enzyme (ACE) as reported for milk protein (Corrêa, Daroit, Coelho, Meira, Lopes, Segalin, Risso, & Brandelli, 2011a; Gonzalez-Gonzalez, Tuohy, & Jauregi, 2011; Kitts & Weiler, 2003; Otte, Lenhard, Flambard, & Sørensen, 2011; Pan & Guo, 2010; Qian et al., 2011; Wang, Zhang, Wang, Feng, & Shan, 2011), soy-protein (Chiang, Tsou, Tsai, & Tsai, 2006; Fung & Liong, 2010; Kodera & Nio, 2006; Nakahara, Sano, Yamaguchi, Sugimoto, Chikata, Kinoshita, & Uchida, 2010; Nakahara et al., 2011; Yeo & Liong, 2010; Zhu et al., 2008), egg protein (Kitts & Weiler, 2003; Yu et al., 2012), fish protein (Harnedy & FitzGerald, 2011), muscle protein (Zhou et al., 2012) and bean protein (Ruiz-Ruiz et al., 2012; Valdez-Ortiz, Fuentes-Gutiérrez, Germán-Báez, Gutiérrez-Dorado, & Medina-Godoy, 2012) and peptides have consequence in the treatment of hypertension. Also, bioactive peptides within the sequence of native proteins have been classically associated with antioxidant activity, and could be used as functional ingredients in food formulations in order to prevent oxidative stress related diseases, and/or to improve the shelf life of foods (Carrasco-Castilla et al., 2011; Corrêa, Daroit, Coelho, Meira, Lopes, Segalin, Risso, & Brandelli, 2011; Kayitesi, De Kock, Minnaar, & Duodu, 2012; Nazeer & Srividhya, 2011; Ruiz-Ruiz et al., 2012; Zhou et al., 2012).

Among these peptides, some contained hydrophobic amino acids (Val or Leu) at the N-terminus, Pro, His, or Tyr in sequences (Chen, Muramoto, & Yamauchi, 1996), and some containing mainly acidic amino acid residues (Glu, Asp) (Xiong, 2010; Zhang, Li, Miao, & Jiang, 2011; Zhou et al., 2012). Metal chelation is partly responsible for the antioxidant activity that has been found in several amino acids, including Tyr, Met, His, Lys, Arg, and Trp (Amarowicz & Pegg, 2008; Blat, Weiner, Youdim, & Fridkin, 2008; Carrasco-Castilla et al., 2012; Xiong, 2010). Because of the side effects of drugs, it is necessary to continue to find new peptides from cheap natural food sources, such as kidney beans, with high antioxidant and ACE inhibitory activity.

1.1.1 OBJECTIVES OF STUDIES.

a. To isolate red kidney bean proteins, separate them into albumin and globulin fractions and study their structural and functional properties.

b. To purify the major storage protein (vicilin) in the globulin fraction followed by structure-function studies.

c. To carry out enzymatic hydrolysis of the globulin proteins, perform size-based separation of the hydrolysate using membrane ultrafiltration and determine the ability of the peptide fractions to inhibit activities of renin, angiotensin converting enzyme and free radicals.

1.2. CONTRIBUTION TO KNOWLEDGE/STATEMENT OF ORIGINALITY

This thesis is intended to represent an original research work that is borne out of independent critical ability to articulate novel research questions which will be answered based on the stated objectives.

The novelty of the research study in objective "a" and chapter 1 is in the fact that, although a plethora of studies have reported on the functional and physicochemical properties of pulses including kidney beans, none has compared the properties of albumin and globulin from kidney bean. Food manufacturers may therefore, find kidney globulin protein as a useful ingredient in the formulation of a variety of specialty foods such as pasta products, ready-to-eat breakfast cereals, baby foods, snack foods, texturized vegetable protein, pet foods, dried soups and dry beverage mixes.

It will also be novel to report the combined effects of varying ionic strength and pH on the circular dichroism (CD) and fluorescence spectroscopy properties of purified kidney bean globular protein (vicilin), since most reports are limited to either the effect of salt or pH and not the combined effect. In addition, a search of literature does not generate published reports on the conformational dynamics of kidney bean oligomeric globulin, as monitored by far-UV CD spectroscopy in the current study. The use of CD to monitor the changes in conformation with change in temperature will help to expand our knowledge on monitoring the effect of heat preparation processes, especially as affected by pH and salt. Chapters 3 and 4 will address these research needs as outlined in objective "b". Also, to our knowledge, different studies performed by different researchers, even with pulses have not reported the antioxidant and antihypertensive potencies of protein hydrolysate from kidney bean. Therefore, novel antioxidant and antihypertensive peptides may emerge if further *in vitro* work is performed with kidney bean globulin protein. Chapter 5 will, therefore report the *in vitro* antioxidant and the antihypertensive properties of kidney bean protein hydrolysate and the ultrafilterated fractions.

1.3. LITERATURE REVIEW

1.3.1. Kidney bean proteins: Kidney bean seeds accumulate large amounts of storage proteins during their development (Duranti, 2006). They are accumulated in membrane-bound organelles, the storage vacuoles or protein bodies, in the cotyledonary parenchyma cells, endure dehydration in seed maturation and undergo proteolysis at germination, thus providing free amino acids, as well as ammonia and carbon skeletons to the developing seedlings (Duranti, 2006). Together, water-soluble albumins and salt-soluble globulins are the main components of kidney bean storage proteins (Sathe, 2002). About 10 to 30% proteins are of the albumin fraction (Kiosseoglou & Paraskevopoulou, 2011), comprising the heterogeneous group of enzymes, amylase inhibitors, and lectins, which are characterized by a relatively low to medium molecular weight and a hydrophilic surface that renders the proteins water soluble (Kiosseoglou & Paraskevopoulou, 2011). The most abundant of the accumulated proteins (45 to 80%) in kidney bean are the globulin proteins because of their globular shapes as well as the requirement of a certain amount of ionic strength for their solubilisation in aqueous media (Sathe, 2002). The globulins (legumin and vicillin) are of high molecular weight and have relatively hydrophobic surface that limits their solubility in water (Kiosseoglou & Paraskevopoulou, 2011). The classifications are based on their sedimentation coefficients (S); for example, 7S and 11S globulins are named vicilin and legumin respectively (Sathe, 2002). Because they are storage proteins, most of them are devoid of any catalytic activity and do not play any structural role in the cotyledonary tissue (Duranti, 2006). In addition, beans also contain some additional proteins such as 17–18S, 11S (very minor), and 2~3S proteins (Sathe,

2002). The 17–18S and 2–3S fractions may contain aggregated and dissociated forms of the main storage proteins, respectively (Sathe, 2002). Under acidic conditions the major protein in bean undergoes reversible association-dissociation between tetrameric (18.2S), protomeric (7.1S), and polypeptide (3.0S) configurations (Sun, McLeester, Bliss, & Hall, 1974). The 2–3S fraction also contains many small proteins, including protease inhibitors and some enzymes (Sathe, 2002). The kidney bean usually contains 20–30% protein on a dry weight basis, and the protein has a good amino acid composition but is low in sulfur-containing amino acids (methionine and cysteine) and tryptophan (Gueguen & Cerletti, 1994; Sathe, 2002). The 7S globulins or vicilins are also sometimes known by several other names such as phaseolin (Osborne, 1894; Waterman, Johns, & Jones, 1923), fraction E (Jaffé & Hannig, 1965), glycoprotein II (Pusztai & Watt, 1970), G1 fraction (Sun & Hall, 1975;Sun et al., 1974), α-component (Ishino & Ortega, 1975), and 7S component (Vaintraub, Bassüner, & Shutov, 1976). The vicilin is a heterotrimeric protein consisting of three polypeptide subunits α-, β- and γ-phaseolin with molecular weight distribution from 43 to 53 kDa (Bollino & Chrispeels, 1978). Vicilins are often glycosylated; they contain carbohydrate residues, such as mannose, glucose, and *N*-acetylglucosamine (Sathe, 2002). The number and extent to which each polypeptide is glycosylated varies according to the type of sugar residues, which explains some of the variability of polypeptide composition of these proteins (Sathe, 2002). This kind of protein shows pH-dependent association–dissociation behavior between tetrameric, protomeric, and polypeptide forms of the molecule (Sun et al., 1974). The 11S proteins are also oligomers, but usually form hexamers (Duranti, 2006). As compared to other *Phaseolus* legumes whose subunits display molecular heterogeneity based on their

differential degrees of glycosylation (Paaren, Slightom, Hall, Inglist, & Blagrove, 1987), kidney bean protein isolate has much higher subunit homogeneity, with a major vicilin content of 83–86%, relative to combined vicilin and legumin (Tang et al., 2008). Therefore, the kidney bean protein isolates may be much more easily processed to perform the role of functional ingredients (Tang et al., 2008). This relative subunit homogeneity gives kidney bean proteins compact structural peculiarity resulting in reduced susceptibility to trypsin digestion as compared to storage protein components from other legumes (DiLollo, Alli, Biliarderis, & Barthakur, 1993; Jivotovskaya, Senyuk, Rotari, Horstmann, & Vaintraub, 1996).

1.3.2. Anti-nutritional compounds (ANCs): Although beans constitute an excellent source of proteins, carbohydrates and fiber and, in addition, provide many essential vitamins and minerals, it is important to note that legumes generally contain a number of toxic or ANCs that may limit their potential health benefits. Duranti (2006) and Siddiq et al. (2011) categorized the toxic substances present into; hydrolase inhibitors and hemagglutinins or "lectins", which are proteinous in nature, and non proteinous ANCs, such as goitrogenic factors, phytates, oligosaccharides cyanogenic glucosides and tannin.

Uebersax et al. (1991) reported that soaking and heating processes improve digestibility of bean proteins in two ways: (1) by denaturation which makes the proteins more susceptible to enzymatic action; and (2) by destroying trypsin inhibitors. In addition, technological processing may promote certain desirable effects like protein coagulation, starch swelling and gelatinization, texture softening and formation of aroma components. Thermal treatment may also improve the nutritional value of food by reducing proteinase inhibition, thus increasing the availability of lysine and other amino

acids. However, the processing conditions applied may induce some detrimental modifications like a loss of vitamins and minerals, the formation of indigestible aggregates and alterations in their conformation (Krupa, Soral-Śmietana, & Lewandowicz, 2007; Linden et al., 2002), as well as causing of crosslinking reactions or amino acids racemization. The impact of phytic acids and tannins can also be reduced/eliminated through thermal processing (Alonso, Aguirre, & Marzo, 2000). Several studies have demonstrated that soaking, cooking, and fermentation of legume seeds reduced the contents of phytic acid, tannins, phenols, α-amylase and trypsin inhibitors (Abd El-Hady & Habiba, 2003; Vidal-Valverde et al., 1997). Shimelis and Rakshit (2007) investigating the effects of different processing and their combinations on the content of ANCs of *Phaseolus vulgaris* varieties, indicated that germination significantly reduced certain heat-stable antinutrient components, whereas cooking of pre-soaked beans appeared to be an adequate method for reducing heat-sensitive antinutrients.

1.3.2.1. Proteinous ANCs:

1.3.2.1.1. The hydrolase inhibitors: The hydrolase inhibitors affect various digestive enzymes, including trypsin, chymotrypsin and amylase (Duranti, 2006), with the most characterized protein inhibitors being trypsin/chymotrypsin inhibitors of both the Bowman-Birk (Domoney, Welham, & Sidebottom, 1993) and Kunitz type (Horisberger & Tacchini Vonlanthen, 1983) as well as α-amylase inhibitors (Moreno, Altabella, & Chrispeels, 1990). Protein fractions that strongly inhibit the activity of enzymes in the intestine reduce protein digestion and hence absorption of their constituent amino acids (Siddiq et al., 2011). Leiner (1975) reported that the proteins of unheated beans resisted

proteolysis in the intestine; after heating, true digestibility increased and trypsin inhibitory activity decreased.

1.3.2.1.2. Lectins: Another family of proteinous ANCs are the lectins or hemagglutinins. Lectins are glycoproteins or carbohydrate-binding proteins which exhibit specific and reversible recognition and binding of diverse sugar structures, and in doing so mediate a variety of biological processes, such as cell–cell and host–pathogen interactions, serum glycoprotein turnover and innate immune responses (Leiner, 1975). These compounds cause agglutination of red cells and impair absorption (Leiner, 1975). However, many of the effects of the proteinous anti-nutritional factors in beans usually manifest only if the seed or the flour is not properly processed (Siddiq et al., 2011). The antinutrients can be eliminated or inactivated to a large degree by appropriate processing treatments (Siddiq et al., 2011). Treatments may include dehulling, presoaking and diffusion, sterilization, steaming and cooking. Wet milling and processing technique employed during protein concentration and isolation also have been reported to be effective in the detoxification of seed materials (Siddiq et al., 2011). Once inactivated, the protein inhibitors may even play a positive nutritional role, due to their high content of sulphur-containing amino acids relative to the majority of the seed proteins (Ryan, Galvin, O'Connor, Maguire, & O'Brien, 2007). Adequate cooking of raw beans can destroy activity of lectins (Shimelis & Rakshit, 2007). Coffey et al. (1985) reported that slow cooking of red kidney beans at 82°C for 160 minutes resulted in a 1-log reduction of bean lectins; this activity was shown to decrease with a linear response at 70°C (Coffey, Uebersax, Hosfield, & Bennink, 1993). Siddiq et al. (2006) reported a significant reduction in lectin activity after 12 hr of soaking (at an initial temperature of 77°C, equilibrating to room temperature at the end of

soak), which can be attributed to higher than ambient initial soak temperature of 77°C. Cooking, after a 12-hr soaking period in boiling water for 45 minutes reduced lectin activity by a total of over 93%, with very little or no differences between 14-, 30-, or 45-minutes cook times.

The undesirable proteinous anti-nutritional components in kidney beans may reduce or limit protein and carbohydrate utilization by humans, but they enable the plant to survive and complete its life cycle under natural conditions. Indeed, due to their anti-nutritional or even toxic properties, various seed components have been shown to play a protective role against insects (Boye et al., 2010), fungi, predators and a number of stress conditions (Duranti, 2006).

1.3.2.2. ANCs of non-protein nature

The antinutritional factors of non-proteinous nature, such as phytates, oligosaccharides cyanogenic glucosides and tannin, cannot be completely inactivated by processing treatments such as soaking and heat. The non-protein compounds like oxalic acid and phytic acid are often heat-stable and, therefore, are the most harmful antinutrients (Siddiq et al., 2011). During cooking however, pectic polysaccharides within the fiber matrix may degrade and become solubilised, thereby reducing the nutritional benefits associated with dietary fiber such as hypocholesterolemic or hypoglycemic effects (Annie & Waldron, 1997; Siddiq et al., 2011).

1.3.3. Glycosylation of storage bean proteins

Storage proteins (vicilin and lectin), all of which are 7S type of proteins are usually glycosylated (Sathe, 2002). The extent and number of polypeptide and sugar moieties involved varies, resulting in differences in polypeptide composition (Sathe, 2002).

Variability in the polypeptides of the glycosylated vicilin-type proteins may be due to insertions/deletions of few nucleotide sequences in the genes encoding these proteins (Schuler, Doyle, & Beachy, 1983). Also, stress factors as well as crossing between wild and domestic types may also contribute to the polypeptide composition variability observed in many varieties of beans (Koenig, Singh, & Gepts, 1990). In beans, glucosamine, xylose, and mannose sugars are often the most common sugars involved in the glycosylation of 7S protein (Sturm, Van Kuik, Vliegenthart, & Chrispeels, 1987). Protein glycosylation is essential for correct folding, transport, and storage of phaseolin and lectin-related polypeptides (Sparvoli, Faoro, Daminati, Ceriotti, & Bollini, 2000), which ultimately affects the structure-function relationship of the protein.

1.4. PROTEIN ISOLATION AND PURIFICATION.

The growing body of research on the health benefits associated with consumption of bean proteins has led to a corresponding growth of interests in developing bean protein-based functional food products. High protein ingredients intended for incorporation as food components or for experimental purposes can be produced in several ways. This includes flour fractions, concentrates and isolates processed by alkali, salt and acid extraction with subsequent purification by chromatographic techniques or ultrafiltration (Uebersax, Ruengsakulrach, & Occena, 1991). For the experimental objectives such as to study functional properties, structure-function properties and biological functions of proteins, it is necessary to remove the major non-protein components such as non bound carbohydrates and fiber that are present in the seeds (Aluko, 2004; Sathe, 2002).

1.4.1. Processing bean protein flours

The cotyledons of the bean seeds must be finely ground to achieve complete cellular disruption and maximum protein and starch separation (Tyler, 1984). The type of milling used must be capable of producing a very fine grind, yet selective enough to break up cells and cell fragments without severely damaging the proteins (Tyler, 1984). After the cotyledon has been ground to a very high degree of reduction in order to achieve complete cellular disruption, proteins can then be extracted by any one or combination of the following techniques; alkaline extraction/isoelectric precipitation, acid extraction, water extraction, salt extraction and ultrafiltration (Boye et al., 2010).

1.4.2. Extraction of legume proteins

1.4.2.1. Alkaline extraction/isoelectric precipitation

Legume proteins can be extracted by aqueous alkaline extraction followed by isoelectric precipitation (IEP) (Boye et al., 2010). The technique takes advantage of the differential solubility of legume proteins, which is high at alkaline pH and low at their isoelectric point, usually pH 4–5 (Boye et al., 2010). In general, ground bean flour is dispersed in water using flour: water ratios ranging from 1:5 to 1:20. The mixture is adjusted to alkaline pH 8–11, which enhance protein solubilization. Thereafter, the mixture is stirred for periods varying from 30 to 180 min to maximize solubilization and extraction of the proteins. During mixing, the pH is maintained at the desired value and temperature may be raised slightly to further enhance protein solubility and extraction. After solubilization, the mixture is filtered to remove insoluble components and pH of the extract adjusted to the isoelectric point to induce protein precipitation; this is followed by centrifugation to recover precipitated proteins, washing to remove salts, neutralization (to enhance

solubility) and drying (Boye et al., 2010). Bean protein fractions extracted using this procedure have shown variability based on the processing conditions such as temperature, time, flour: solvent ratio, condition and protein solubility of the starting material, type of equipment and process used, *g* forces used for centrifugation, laboratory vs. pilot- scale extraction, batch vs. continuous extraction, etc (Boye et al., 2010). Chakraborty et al. (1979) isolated proteins from some pulses at pH 8.5 (30 min, 40^{o}C) followed by precipitation at pH 4.5. The protein contents obtained were respectively, 90.5%, 90.1%, 91.2%, 89.3%, 91.2% and 88.3% on dry weight basis, for chickpea, pea, great northern bean, lentil, lima bean and mung bean. Paredes-Lopez et al. (1991) isolated protein from a defatted chickpea using similar conditions as Chakraborty et al. (1979) and obtained a product with lower protein content (84.8% on dry weight basis), implying the effect of defatting on the percentage protein recovered. Fernandez-Quintela et al. (1997) obtained 81.2% and 84.9% protein contents on a dry weight basis, respectively from de-hulled faba bean flour and pea flour, when the flours were dispersed in water (1:5 w/v bean flour to solvent ratio, pH 9, 20 min, room temperature); this shows how temperature differences may affect protein recovery. Flink and Christiansen (1973) made protein isolates from seeds of *Vicia faba* using a 1:5 (w:v) bean flour to solvent ratio, pH 8–10, 23^{o}C, 10 min, followed by centrifugation and precipitation of proteins from the supernatant at pH 3.5. The protein contents of the isolates ranged between 80% and 90%. A slightly modified method of Flink and Christiansen (1973) was used by McCurdy & Knipfel, 1990 (extraction at pH 7–10, 30 min, 10 and 20^{o}C, 1:5 bean to solvent ratio, with or without 0.3 M NaCl, followed by precipitation at pH 4–5.3) to produce isolates with protein contents ranging from 76.4% to 94.0%.

1.4.2.2. Acid extraction

The acid extraction is based on the principle that is similar to that of alkaline extraction. Like alkaline extraction, processing conditions used for acid extraction can influence the yield and purity of the finished product (Boye et al., 2010). The difference is that the initial protein extraction is conducted under acidic conditions. At pH <4 (acidic condition), solubility of bean proteins increased, making it possible to solubilize proteins prior to their recovery by isoelectric precipitation, cryo-precipitation or membrane separation (Boye et al., 2010). A citric acid solution (0.4 N, pH 4.0) was used to prepare protein isolate from ground white kidney bean (*Phaseolus vulgaris*) followed by refrigeration (4^{o}C, 18 h) to precipitate the protein material with 95.7% protein and 2.6% total carbohydrate contents, on a dry weight basis (Alli, Gibbs, Okoniewska, Konishi, & Dumas, 1993). Vose (1980) obtained protein contents of 91.2% and 91.9% for faba bean and pea, respectively, by direct acidification (pH 4.4–4.6) of the supernatant from starch extraction of pin-milled faba bean and pea. With a slightly different method, Ologhobo et al (1993) isolated a product with 50% protein content (based on dry weight) when lima bean was suspended in flour (flour to water ratio of 1:10) at pH 4.5 (8 h, room temperature) followed by centrifugation, re-extraction, and dialysis.

1.4.2.3. Organic acid extraction

Sometimes organic solvents are used for the extraction of proteins (Doonan, 2004). The principle behind the use of organic solvents includes the decrease in dielectric constant, which promotes aggregation by charge interaction, as well as sequestration of water of solvation of the protein. The problem with organic solvents is protein denaturation by interaction with hydrophobic residues in the protein interior . It is therefore, essential to

carry out solvent precipitation at a low temperature to minimize denaturation. The solvents used for the precipitation of proteins need to be miscible with water in all proportions and should be nontoxic.Acetone and ethyl alcohol best meet these requirements (Doonan, 2004).

1.4.2.4. Salt and water extraction

Osborne (1909) precipitated proteins from a range of plant sources, including the major storage proteins of cereal and legume seeds. He classified four groups that were extracted sequentially in water (albumins), dilute salt solutions (globulins), alcohol–water mixtures (prolamins), and dilute acid or alkali (glutelins). This classification still forms the basis for current studies of seed storage proteins. The most widely used procedures for the precipitation of albumin and globulin proteins in legume are respectively, by water and by the addition of salt (based on the salting-in and salting-out phenomenon of food proteins).

1.4.2.4.1. Water extraction: Bean proteins may be extracted with water without subsequent acid precipitation (Boye et al., 2010). Proteins were extracted from seed of five mature dry bean cultivars (*Phaseolus vulgaris*) using a two-time extraction with water (Martin et al., 1995) with seed flour: water ratio of 1:10 at 1–4^{o}C. The mixture was allowed to stay for 16 h, and the protein content was then measured using the supernatants ranged from 5.5–10 g N/kg seed meal. Cai et al. (2001) also did a two-time extraction of proteins from chickpea, common bean, faba bean, lentil, mung bean and smooth pea by blending 200 g of each seed with 500 mL of water. The protein contents of the first extracts ranged from 54% for chickpea to 67% for smooth pea. After the

second extraction, the highest protein content of 65.6% was obtained for faba bean and the lowest for chickpea flour with 50.5% protein.

1.4.2.4.2. Salt extraction: The addition of a high amount of salt to a protein solution causes precipitation by the removal of water of solvation from non-polar patches on the protein's surface, thus allowing these patches to interact with resulting aggregation (Doonan, 2004). Osborne (1894), supported by other authors (Joubert, 1957; Waterman et al., 1923), explained that some of the extractable proteins in legume seeds are salt-soluble. Aluko (2004) gave further details of the observation of these pioneer authors, that, solubility of a protein is dependent on salt concentration. For example, at low ionic strength, the solubility of proteins increases with the salt concentration but at high ionic strength, protein solubility decreases. Englard & Seifter, (1990) reported that, for a pure protein, the relationship between solubility, *S* (in g/kg of water) and the ionic strength, *I* (in mol/kg water) is given by:

$$\log S\text{-}b\text{-}Ks[(I/2)]$$

Where b and *Ks* are constants for a particular protein at a particular pH and temperature. The argument is that a protein will precipitate over a range of ionic strength values (determined by the value of *Ks*) and that different proteins will precipitate over different, but frequently overlapping salt concentrations (Englard & Seifter, 1990).

A variety of salts has been used for protein extraction, including NaCl, Na_2SO_4, KCl, $CaCl_2$, and $MgSO_4$, and these are still sometimes used for particular applications (Doonan, 2004). By far the most frequently used salt is ammonium sulfate ($[NH_4]_2SO_4$) (Doonan, 2004). The reasons for this include: a) high solubility in water (about 4 M at saturation), b) low heat of solution, c) density of saturated solutions (1.235 g/mL) is less

than that of proteins, hence allowing for collection of precipitated proteins by centrifugation, and d) the ions have no negative effects on proteins (Doonan, 2004). Based on this principle and the inherent advantages that ammonium sulfate has been used to precipitate/fractionate the major protein fractions in cowpea (Rangel, Domont, Pedrosa, & Ferreira, 2003), defatted canola meal (Wu, Aluko, & Muir, 2009) mungbean flour (Mendoza, Adachi, Bernardo, & Utsumi, 2001) and kidney bean (Jaffé & Hannig, 1965; Mikkonen, 1992; Shi, Xue, Kakuda, Ilic, & Kim, 2007). Once extracted, they are centrifuged to separate the solubilized proteins from insoluble materials (Aluko, 2004). The supernatant may then be dialyzed against distilled water to precipitate the salt soluble globulin from the water soluble albumin (supernatant), or by the use of buffer exchange on Sephadex G25, or even by using hydrophobic interaction chromatography (Aluko, 2004). The resulting protein fractions can be further purified by various separation methods based on their molecular properties such as size, hydrophobicity, ionic properties, and affinities for certain ligands (Aluko, 2004).

1.4.3. Purification of bean proteins

After extraction and precipitation, one or more chromatographic or electrophoresis techniques may be used to purify the protein of interest so as to obtain a homogeneous preparation (Aluko, 2004). Protein purification is essential in understanding molecular characteristics such as functional, structural and conformational as well as bioactive properties. Bonner (2007) summarised some of the reasons for protein purification as:

1) To remove conflicting enzyme activities that may be present in a crude extract.

2) To establish the effects of activators and inhibitors on functions of a protein.

3) To enable the determination of the molecular mass and post-translational modifications.

4) To determine partial sequence of proteins; this information can then be used to identify the responsible gene.

5) The purified protein can be used to grow crystals for detailed structural studies.

6) *Antibodies* can be raised against a purified (or partially purified) protein which can be used to determine cellular location or cross-reactivity with different species.

The following basic rules have been proposed by Aluko (2004) as essential in protein purification:

1. Partitioning should be based on the differences in surface charge, surface hydrophobicity, molecular weight, isoelectric point values, biospecificity to ligands, temperature and pH values.

2. Eliminating unwanted compounds (including proteins and other compounds) that are found in greater percentage first.

3. Using a high resolution purification step as soon as possible.

The overall essence of any chromatographic technique is to show high capacity, good resolution and produce good yield for protein purification. One technique demonstrating these properties is fast protein liquid chromatography.

1.4.3.1. Fast Protein Liquid Chromatography (FPLC): FPLC is designed to separate proteins in their native active configuration, and proteins separated by this technique in the laboratory can easily be scaled up. FPLC provides a full range of chromatography modes, such as ion exchange, chromatofocusing, gel filtration, hydrophobic interaction, and reverse phase, based on particles with average diameter sizes of as small as 5–40 μm

(Aluko, 2004; Bonner, 2007). A brief explanation of the working principle of some forms of FPLC as an example of chromatographic technique is given below:

1.4.3.2. Size exclusion chromatography (SEC). Also known as gel filtration, gel permeation and molecular sieve, SEC involves separation of protein molecules based on their size and shape. The column is packed with beads, which are made up of insoluble but highly hydrated polymers that allows a partition of molecules between two liquid volumes; the volume of the mobile phase and the accessible volume contained within the stationary porous bead (Aluko, 2004; Bonner, 2007). The separation in SEC does not involve binding between the sample and the resin and hence proteins can be separated in their native conformation with good recovery of biological activity.

1.4.3.3. Ion exchange chromatography (IEC) Ion exchangers bind charged molecules, and there are essentially only two types of ion exchangers, anion and cation. The net charge of a protein depends on the pH; positive at very low pH, negative at high pH, and zero at some specific points in between, which is termed the isoelectric point (PI). Ion exchangers consist of immobilized charged groups and attract oppositely charged proteins. They provide the mode of separation that has the highest resolution for native proteins. High-performance reversed phase chromatography has equivalent or even better resolution, but it generally involves at least partial denaturation during adsorption and so is not recommended for sensitive proteins (Aluko, 2004; Bonner, 2007)

1.4.3.4. Hydrophobic-interaction chromatography (HIC): The working principle of HIC is based on the fact that, all proteins are to an extent hydrophobic (Bonner, 2007). Therefore, HIC is used to separate proteins on the basis of the differences in the strengths of hydrophobic interaction between the proteins and a matrix containing immobilized

hydrophobic groups. Hydrophobic amino acid residues with nonpolar R groups such as alanine, phenylalanine, valine, tryptophan, leucine, isoleucine, and methionine are usually buried within the protein molecule. However, some nonpolar amino acids are found on the surface of the molecule as distinct hydrophobic patches because of constraints in folding. These patches of hydrophobic amino acids are important in the maintanance of protein structure, and thus in determining the biological function of the molecule. Since the number of hydrophobic patches and their degree of hydrophobicity is characteristic of a particular protein, it becomes possible to selectively adsorb and elute proteins to and from a suitable hydrophobic matrix. Proteins are loaded onto a matrix containing hydrophobic ligands in a high-salt environment, and then eluted by decreasing the salt in the environment or by some other method of adjusting the polarity of the water phase (e.g., by adding nonionic detergents or organic solvents) (Aluko, 2004; Bonner, 2007).

1.4.3.5. Chromatofocusing (CF): As a chromatographic technique, CF separates proteins on the basis of their isoelectric points. CF is useful for the purification of proteins with isoelectric points in the range of pH 3 to 11. Essentially, CF is an ion-exchange technique whereby proteins are bound to an anion exchanger and then eluted by a continuous decrease of the buffer pH (Aluko, 2004; Deutscher, 1990). Proteins elute from a CF column in descending order of isoelectric point. Appropriate pH gradient range is first determined on the basis of the isoelectric point of the target protein, followed by selection of elution buffers (Deutscher, 1990; Aluko, 2004; Bonner, 2007).

1.4.4. Electrophoresis of proteins

Throughout the purification process the purity of the protein of interest must be regularly monitored. Denaturing polyacrylamide gel electrophoresis (PAGE) is the most widely used technique to monitor the purity of pooled protein fractions after chromatography (Berkelman & García-Carreño, 2001). This technique denatures proteins with a detergent such as sodium dodecyl sulfate (SDS) and a reducing agent, e.g. 2-mercaptoethanol (2-ME) into polypeptides and then separates denatured and fully reduced proteins on the basis of their molecular weight. Alternatively, native discontinuous PAGE is used for separation. Under native conditions, polypeptides retain their higher-order structure and often retain enzymatic activity and interactions with other polypeptides. The migration of proteins under native conditions depends on many factors including size, shape, and charge. For native electrophoresis, no SDS or any other reducing agent (DTT or 2-mercaptoethanol) is added. A homogeneous band on both denaturing and nondenaturing PAGE is a good indication of the purity of the protein fraction. Isoelectric focusing (IEF), capillary electrophoresis (CE), or reversed phase chromatography (RPC) are other techniques which could be considered to supplement denaturing SDS-PAGE (Berkelman & García-Carreño, 2001).

1.4.5. Ultrafiltration: Ultrafiltration is a pressure-driven barrier membrane separation. The membrane is usually carefully selected with specific molecular weight cut-offs to enable retention of proteins or peptides of interest. Factors such as the molecular weight cut-off, type of membrane and the volume concentration ratio and diafiltration conditions affect efficiency of separation of the proteins. Using this process, alkaline or acid extracted supernatant or enzymatic hydrolysate can be extracted (Boye et al., 2010). Vose

(1980) used ultrafiltration to prepare a protein isolate from faba bean and pea flour with 94.1% and 89.5% protein content, respectively.

1.5. FUNCTIONAL PROPERTIES OF BEAN PROTEINS

Proteins play an important role in the manufacture of food products. Intrinsic physicochemical properties that influence the behavior of proteins in food systems during processing, manufacturing, storage and preparation include a) hydration-related properties such as solubility, wetability, swelling, water absorption/adsorption, thickening, gelling, b) surface properties e.g. emulsion, foaming (aeration/whipping), protein-lipid interactions, film formation, lipid and flavor binding, and c) structural/rheological properties comprising elasticity, grittiness, cohesiveness, chewiness, aggregation, gelation, stickiness, viscosity, texturization, fiber formation, dough-forming ability, extrudability, and adhesion (Sathe, 2002). In response to changes in processing environments (e.g. changes in pH and ionic strength) and composition, proteins individually and in combination with other ingredients can cause meaningful interactions that lead to diversified functional properties in the end-products (Boye et al., 2010; Kinsella, 1979).

For their high nutritional value and minimum cost, beans, including kidney beans are exploited as functional ingredients for the preparation of various meals, either on their own or as ingredients in other food materials. When these beans are used as ingredients in other food materials, the main target is to prepare meals that have superior nutritional and organoleptic properties than the raw materials. Protein molecules in beans interact with other food ingredients to affect macroscopic properties such as physical stability, texture and mechanical characteristics (Boye et al., 2010; Siddiq et al., 2011). The molecular

properties of food proteins including hhydrophobicity, hydrogen bonds, ionic forces, and covalent bonding are involved in these interactions with other components. These interactions within the food system ultimately affect the behaviour of proteins during preparation, processing, storage, and consumption (Kinsella, 1979; Sathe, 2002). These functional properties make it possible to manufacture products of desirable quality and can be improved by intentional enzymatic or chemical modifications of the protein molecules. Various studies have increased our understanding of the functionality of pulse proteins such as Lima bean globulin protein (Chel-Guerrero, PÃrez-Flores, Betancur-Ancona, & DÃvila-Ortiz, 2002), yam bean (Mélo, Stamford, Silva, Krieger, & Stamford, 2003), faba bean, pea and soybean (Fernandez-Quintela, Macarulla, del Barrio, & Martinez, 1997) and kidney beans (Chau & Cheung, 1998; Chau, Cheung, & Wong, 1997; Tang & Ma, 2009; Yin, Tang, Wen, Yang, & Li, 2008).

1.5.1. Solubility: The percentage of protein contained in the food material that can be extracted by water or a suitable solvent under specified conditions is its protein solubility (PS). PS depends on a number of factors including specific properties of the protein and of the solvent, pH, concentration and charge of other ions, ratio of sample weight to solvent volume, particle size of the sample, duration of extraction, and temperature (Sikorski, 2006). Depending on the number and identities of the charged amino acids, and on pH, the surfaces of amino acid residues in proteins are either rich in ionizable residues such as arginine, lysine, aspartic acid, and glutamic acid or abundant in hydrophobic groups (Sikorski, 2006). Proteins rich in ionizable residues and low in surface hydrophobicity are soluble in water or dilute salt solutions, for example, the proteins found in egg white. In contrast, proteins with abundant hydrophobic groups readily

dissolve in organic solvents or may undergo protein-protein interactions and precipitation when in aqueous medium (Sikorski, 2006).

For proteins that are soluble in aqueous solvents, the solvent pH determines net charges on the protein. At neutral pH for example, aspartic acid and glutamic acid carry a negative charge and arginine and lysine carry positive charges. At the isoelectric point, the positive and negative charges will balance each other, the net charge will be zero and PS will be at its lowest point. When pH of the solvent becomes more alkaline, lysine and arginine residues lose their positive charge and become neutral at pH 12. If the pH is made more acidic, however, aspartic acid and glutamic acid shed their negative charge by acquiring protons and become neutral below pH 4.0 (Sikorski, 2006). Legume globulins, which constitute the main proteins of concentrates or isolates prepared from legumes, contain more hydrophobic groups in nature and tend to exhibit reduced solubility at pH environments close to the isoelectric point, where electrostatic repulsion and ionic hydration of molecules reach a minimum (Kiosseoglou & Paraskevopoulou, 2011).

In general, the solubility of legume protein materials (flours, concentrates or isolates) is very low in the range between pH 4 and 6 but exhibits a sharp rise when the pH is moved either to more acidic or to neutral and alkaline environments (Kiosseoglou & Paraskevopoulou, 2011). Protein functional properties, such as emulsification, foaming or gelation, are closely related with solubility, which may determine to a large extent the protein suitability as an ingredient in various food applications. The solubility against pH curve can be used for selecting parameters for extraction of proteins from different sources (Sikorski, 2006).

1.5.2. Water- and oil-absorption capacity (WAC & OAC): WAC and OAC are, respectively, the amount of water and oil that can be held per unit weight of the protein material (Sikorski, 2006). WAC and OAC make up useful indicators of the ability of proteins to inhibit fluid leakage from a product during food storage or processing. WAC values ranging from as low as 0.6 to as high as 4.9 g g^{-1} have been reported in a number of studies (Kiosseoglou & Paraskevopoulou, 2011) for protein isolates or concentrates prepared from legumes such as chickpea, kidney bean, pea, faba bean or lentil, indicating that both the type of pulse and variety may have an effect on WAC. In addition to the influence of the type of pulse on WAC, the technique employed for protein extraction may also influence the WAC value, with the protein materials obtained by isoelectric precipitation, for example, from pea or chickpea exhibiting higher water-binding ability than those prepared by ultrafiltration (Boye et al., 2010). As well, OAC values reported for protein materials prepared from various pulses range from 1.0 to 3.96 g g^{-1} depending on the type of legume, legume variety and method of preparation (Kiosseoglou & Paraskevopoulou, 2011). WAC and OAC data are useful for assessing the technological suitability of bean protein materials in food applications.

1.5.3. Emulsifying and foaming properties

When oil is homogenized with protein solutions, oil–water interface and oil droplet formation takes place, usually sustained by adsorption of protein molecules to the newly formed oil droplet surfaces. The initially formed surface layer at the droplet surfaces is further strengthened as new protein molecules adsorb and rearrange to a state of minimum free energy and, at the same time, network with other adsorbed molecules leading to the development of a mechanically strong protein layer. Proteins that are

adsorbed to the surfaces help to form, stabilize and protect emulsions from immediate coalescence into larger ones. The emulsifying properties of proteins are often expressed as emulsifying activity index (EAI) and emulsifying stability index (ESI) (Boye et al., 2010). The EAI measures the maximum surface area created per unit protein i.e. the amount of oil that can be emulsified per unit of protein, whereas ESI evaluates the ability of the emulsion to resist changes to its structure over a defined time period, which defines the ability of a protein to stabilize an emulsion (Boye et al., 2010). The ability of a protein to aid in the preparation of an emulsion depends on the molecular characteristics of the protein such as size, solubility, surface hydrophobicity and structural flexibility, as these characteristics determine the protein adsorption properties. The emulsifying properties of pulse proteins are also dependent on environmental parameters such as pH and ionic strength. Zhang et al. (2009) observed that a chickpea protein isolate obtained by isoelectric precipitation exhibited higher EAI at alkaline pH than at a pH close to the protein isoelectric point, where emulsifying ability of the protein dropped dramatically. In addition, the EAI of chickpea protein decreased at relatively low ionic strength environments (around 0.1) and increased again as the ionic strength of the protein solution was increased. These differences in EAI were related to surface hydrophobicity and molecular secondary structure changes brought about by pH and ionic strength manipulation. Based on the determination of EAI, a number of studies reported that the emulsifying ability of pulse protein concentrates or isolates is affected by the type of beans or the method applied for their preparation. For example, according to Fuhrmeister and Meuser (2003), a pea protein isolate prepared by isoelectric precipitation exhibited lesser emulsifying ability compared to the respective isolate obtained by ultrafiltration

(EAI values of 10.1 or 14.0 $m^2 g^{-1}$ and 27.4 $m^2 g^{-1}$, respectively). Boye et al. (2010) compared the EAI and ESI of a number of protein concentrates with a protein content ranging between 63.9 and 88.6%, obtained from pea, chickpea or lentil by applying isoelectric precipitation or ultrafiltration. These researchers deduced that the emulsifying properties of the proteins depended mainly on the type of pulse used for protein concentrate preparation, with the protein concentrates from chickpea showing higher emulsifying ability compared to those of pea or lentil, while the method applied for the preparation of the concentrates had minimal impact.

1.5.4. Foaming properties

Foams are formed when proteins form an interface that keeps air bubbles in suspension and prevents their collapse. Often foaming properties of proteins are measured by three indices, namely, foam expansion (FE), foam capacity (FC) and foam stability (FS) (Boye et al., 2010). The vital requirements for a protein to be a good foaming agent are the ability to: (a) adsorb promptly at the air/water interface during bubbling, (b) undergo rapid conformational change and rearrangement at the interface and (c) form a cohesive viscoelastic film via intermolecular interactions (Sánchez-Vioque, Clemente, Vioque, Bautista, & Millán, 1999). Foam formation of proteins is a very important characteristic that is widely employed in beverages, mousses, meringue cakes and whipped toppings. Foam stability requires the specific properties of protein films such as formation of cohesive, viscous, elastic, continuous, air-impermeable film around each gas bubble. Film thickness, mechanical strength, protein-protein interactions and environmental factors such as pH and temperature have very strong influence on foam stability (Sai-Ut, Ketnawa, & Rawdkuen, 2009). Generally, globular proteins which are the dominant

proteins in legume foods are relatively resistant to surface denaturation and hence give low foamability. The lower FC of globulin proteins may be due to reduced ability of the globular proteins to unfold at the air-water interface, which limits capacity to encapsulate air bubbles (Sai-Ut et al., 2009).

1.6. EFFECT OF SUBSTITUTION OF DRY BEAN FLOURS ON THE FUNCTIONAL PROPERTIES OF OTHER PRODUCTS

Legume seeds and ingredients contained in them have been utilized in the preparation of new or improved food products, in particular bakery products, soups, extruded products, ready-to-eat snacks, infant formulas and dietetic foods. Taking advantages of the good functional properties (solubility, foaming, water- and fat-binding capacity), a well-balanced content of essential amino acids (especially when consumed with cereals and food rich in S-amino acids and tryptophan), and high protein concentrates (>65% protein) and isolates (>90% protein), dry beans have been used as ingredients in various food products. For instance, chickpea, lentil, pea, navy bean and kidney bean flours and their proteins have been used to prepare gluten-free crackers with good consumer acceptability (Han, Janz, & Gerlat, 2010). Spaghetti fortified with 35% bean flour has been developed (Petitot, Boyer, Minier, & Micard, 2010). Dry bean flours can partly take the place of wheat flour in bakery products, pasta, noodles, crackers and snack foods (Anton, Ross, Lukow, Fulcher, & Arntfield, 2008; Gómez, Oliete, Rosell, Pando, & Fernández, 2008; Hood-Niefer & Tyler, 2010; Petitot et al., 2010; Sabanis, Makri, & Doxastakis, 2006; Wood, 2009). Dry bean flours also have potential for use in prepared meat systems,

batters and coatings, beverages, yogurt, salad dressings and desserts (Wood & Malcolmson, 2011)

Deshpande et al. (1983) showed that substitution of whole bean (*Phaseolus vulgaris*) flours increased water and oil absorption, and foaming and emulsification capabilities when compared to 100% wheat flour. Pasting and mixing properties were also altered with the addition of bean flours. Interestingly, it has also been shown that, the flour from kidney bean showed higher functional properties, e.g., gelation capacities, emulsifying activity and emulsion stability, relative to soybean flour (Chau & Cheung, 1998). It was also shown that the vicilin (or phaseolin) from kidney beans exhibited best emulsifying ability, when compared to pea, faba bean, cowpea and soybean vicilins (Kimura, 2008). Previous work also showed better gelling property for kidney bean protein isolate as compared to red bean, and mung beans (Tang, 2008). These good properties of the flours as well as the isolates are largely contributed by the proteins constituents, which make both the kidney bean flour and protein isolate exhibit good potential to be applied as an excellent functional food ingredient. The surface active properties (e.g., foaming and surface hydrophobicity) of proteins from kidney beans were however different depending on the method of preparation. The functional properties of bean flours are critical to the quality characteristics of the final food product. Therefore, care must be taken in the production of the flour to ensure that the desired functional properties (such as water and fat absorption, protein solubility and starch gelatinization) are achieved; otherwise processing and end-product quality will be compromised.

1.7. STRUCTURAL CHARACTERIZATION OF BEAN PROTEINS

Globular proteins have compact, tightly folded structures in their native state. However, a change in environment or treatment such as subjecting them to extremes of pH, high temperatures, detergents, and solutions containing high concentrations of compounds such as urea, guanidinium chloride, and organic solvents will cause the protein to unfold (Katta & Chait, 1991). The physicochemical properties of the unfolded proteins are usually different than those of the folded molecules (Katta & Chait, 1991). Protein unfolding affects all biological processes including catalysis, transport, regulation, defense, and maintenance of cell architecture, which depend on the stability and the appropriate conformation of the proteins (Pain, 2003). It is therefore important to understand how proteins fold into biologically active states and how these active states are stabilized. After protein purification, there is a need to perform structural studies under the conditions in which bean proteins actually operate (i.e., generally in solution), as well as under other conditions and to provide measures of the rates of structural changes of proteins, which are often essential to their biological function (Pain, 2003). Techniques, including optical rotation, spectrophotometry, viscometry, fluorescence, circular dichroism, and nuclear magnetic resonance (NMR) have been applied to monitor these conformational changes (Pain, 2003). Spectroscopic methods such as circular dichroism (CD) and fluorescence spectroscopy (FS) are powerful analytical tools that can measure conformational changes that may occur (at the molecular level) due to changes in the protein environment.

1.7.1. Circular dichroism (CD) spectroscopy:

CD spectroscopy has a good precision for assessing changes in both the secondary and tertiary structure of proteins, as well as provides information regarding prosthetic groups, bound ligands and co-factors (Kelly & Price, 2006; Pain, 2003). CD spectroscopy can be used to measure changes in stability of the protein upon complex formation as a result of thermal or chemical denaturation, even when the change is minimal (Kelly & Price, 2006). Mean residue ellipticity, which is the molar ellipticity of the protein divided by the number of residues, is the standard unit of reporting CD spectroscopy measurement (Kelly & Price, 2006). Signals arising from chromophores, including the amide group of the polypeptide backbone with associated peaks falling in the far UV region (190-240nm), and the aromatic residues (Phe, Tyr, and Trp) around the near UV region (250-320 nm) of the spectrum, makes CD spectroscopy suitable for examining protein conformation and folding under a wide range of experimental conditions (Kelly & Price, 2006; Pain, 2003). Changes in the near ultraviolet (UV) CD spectra of proteins imply changes in tertiary structure. Protein-protein or protein-ligand interaction may result in significant changes in tertiary structures of one or all components upon complex formation leading to significant changes in the near-UV CD spectrum. The signals from the near-UV CD spectra is usually associated with the presence of aromatic amino acid side chains or a particular non-protein cofactor, since these will provide convenient signals for monitoring the interaction between proteins (Kelly & Price, 2006). Conversely the far UV provides information about the polypeptide backbone and its conformation typically reflecting the secondary structure of the protein (Pain, 2003).

The presence of a chromophore and the closeness of the chromophoric group to an optically asymmetric environment are the two requirements for a molecule or group of atoms in a protein to exhibit a CD spectrum (Pain, 2003). CD spectra are specific to the particular environmental influences acting on the chromophore and their interaction with other neighboring chromosphores (Pain, 2003). From the intensity and appearance of the CD spectroscopy signals, analysts can draw conclusions about the changes in conformation of the protein or environment of the components. The advantages of CD measurement as a spectroscopic technique is the ability to use it to study a wide range of interactions by monitoring changes in signal from either the protein or the ligand (or both) (Kelly & Price, 2006). Also, changes in the CD signals in structural terms, e.g., in terms of the secondary structure of the protein (far-UV) or the integrity of the ligand-binding site can be interpreted (Kelly & Price, 2006). In addition, the interactions can be studied under varying experimental conditions such as protein concentration, pH, temperature, ionic strength, etc. (Kelly & Price, 2006)

1.7.2. Fluorescence Spectroscopy (FS)

Proteins are capable of emitting fluorescence which can be measured as a signal, when excited with ultraviolet light (Pain, 2003). This fluorescence signal is characteristic of the protein and very environmentally sensitive, thus making this method useful for gaining information about protein structures (Pain, 2003). Protein fluorescence can reveal a variety of information, including the extent of rotational freedom, the exposure of amino acid side chains to quenchers, and intramolecular distances (Pain, 2003). Therefore, changes in intrinsic fluorescence can be used to monitor structural conformations of proteins. The emission maximum of the intrinsic fluorescence amino acids

(phenylalanine, tyrosine and tryptophan) in their specific environment could be a useful signature because it provides a sensitive means of characterizing proteins based on their conformation, dynamics, and intermolecular interactions (Kelly & Price, 2006; Pain, 2003). If the protein does not contain large amounts of intrinsic aromatic amino acids and hence, it is not able to provide sufficient signal for the monitoring of the interaction, an extrinsic label (fluorescent dyes) should be attached at an appropriate position within the protein (Kelly & Price, 2006). Extrinsic labels have a much higher quantum yield, and hence may allow the use of much lower protein concentrations (Kelly & Price, 2006). Generally the main advantage of fluorescence is its requirement for very limited quantities of material; only a nanomole of the analyte is required (Kelly & Price, 2006).

1.8. BIOLOGICAL ACTIVITIES OF PROTEINS FROM BEANS

Worldwide, epidemic of chronic diseases account for 58 million premature deaths annually, and it's been projected that by 2020, chronic diseases will account for almost three-fourths of all deaths (Mine, Li-Chan, & Jiang, 2010; Yach et al. 2004). Dietary modification, in particular, therapeutic diets including high amounts of vegetables, fruits, legumes, whole grains, low-fat dairy foods, low amounts of saturated fats and salt and isolated food proteins and peptides have indicated beneficial effects on several chronic diseases (Mine et al., 2010). Specifically, scientific evidence concerning certain biological activities of food proteins and peptides is growing (Hartmann & Meisel, 2007; Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008). Health effects attributed to food-derived peptides includes, cholesterol-lowering ability, antimicrobial properties, blood pressure-lowering (ACE inhibitory) effects, antithrombotic and antioxidant activities,

enhancement of mineral absorption and/or bioavailability, cyto- or immunomodulatory effects, and opioid activities (Hartmann & Meisel, 2007; Möller et al., 2008). Functional protein and peptide products already on the market which have scientifically evidenced health claims are currently valued at $75 billion/year (Mine et al., 2010).

Legumes are important sources of dietary proteins and bioactive peptides. There has been increased interest in their beneficial effects in terms of several health claims, such as, opiate, immunomodulation, angiotensin-I-converting enzyme (ACE) inhibiting, antithrombotic, antioxidant, and antimicrobial activities (Sirtori, Galli, Anderson, & Arnoldi, 2009). Important biological activities have been described for 7S globular proteins from various beans, especially lectins and enzyme inhibitors. Reports have shown that, although protease inhibitors, amylase inhibitor and lectins have long been considered as anti-nutrients, these proteins exert beneficial effects at low concentrations. Most studies on the potential impact of specific legumes on cardiovascular disease-related endpoints have focused on soybeans. For instance, Lovati et al. (1996) reported the involvement of 7S globulin family of soybean storage proteins in the 35% reduction of plasma cholesterol levels with statistically significant decrease in triglyceride levels in rats. Subsequently the α′ subunit of the 7S globulin was shown to be responsible for this activity (Lovati, Manzoni, Gianazza, & Sirtori, 1998; Lovati et al., 2000). The oral administration of affinity chromatography purified α′ subunit of the 7S globulin oligomer to animal models confirmed significant reduction of plasma cholesterol and triglyceride levels (Duranti et al., 2004; Manzoni et al., 2003). Moreover, a further test with the α′ subunit of the 7S globulin in isolated cell systems ruled out any isoflavone effect (Duranti et al., 2004), and substantiated the role of a legumes dietary protein in the management of

dislipidemia. The evidence was assessed to be so strong that the American Food and Drug Administration authorized a specific health claim that associates soy consumption to the prevention of heart disease in 1999 (Food and Drug Administration, 1999). Other authors have reported formulation of therapeutic products from legume proteins and peptides for treatment and prevention of human diseases. In particular, antidiabetic and antiobesity role for α-amylase inhibitor from kidney bean (*P. vulgaris* L.) has been reported (McCarty, 2005; Obiro et al., 2008). An inhibitory activity on tumor growth has been demonstrated for lectins from several plant sources, including kidney beans (Pryme, Bardocz, Pusztai, & Ewen, 2006). Also, lectins from bean seeds have been shown to exert a potent antihepatoma effect, being a potential therapeutic agent for liver tumor (Lei & Chang, 2009). Anticarcinogenic effects of the trypsin and chymotrypsin inhibitors of the Bowman-Birk class have been described not only from soybean, but also from pea seeds. Soybean protease inhibitors are active against colon, liver, lung, esophagus and breast cancers, while pea protease inhibitors showed very high antiproliferative activity on human colon cancer cells (Clemente, Gee, Johnson, Mackenzie, & Domoney, 2005; Clemente & Domoney, 2006). Animal models have clearly shown that these inhibitors are absorbed after oral administration and are found in liver, kidney and lung in an intact form (Wan et al. 2002). Bioactive peptides are resistant to the action of peptidase and act as physiological modulators during gastrointestinal digestion of soy products. They stimulate superoxide anions, which trigger non-specific immune defense systems (Kitts & Weiler, 2003) and show antioxidant, antiobesity and anticancer activities (Peñta-Ramos & Xiong, 2002).

1.8.1. Antioxidant effects of legumes: Reactive oxygen species (ROS), for example, hydroxyl radicals (•OH), peroxyl radicals (•OOR), superoxide anion ($•O_2$), and peroxynitrite (ONOO), attack proteins, lipids, and nucleic acids resulting in cell damage and apoptosis, which plays an important role in atherosclerosis, Alzheimer's disease, inflammatory bowel disease, and certain cancers (Stadtman, 2001). The endogenous antioxidant systems, including enzymes (superoxide dismutase, catalase, and glutathione peroxidase), and various nonenzymatic compounds (selenium, α-tocopherol, and vitamin C) help to keep the health of cells, tissues, and organs of humans under normal physiological conditions (Xiong, 2010). Amino acids (e.g. histidine, cysteine, methionine, and tyrosine), peptides (e.g. glutathione, carnosine, and anserine), and proteins also contribute to the overall antioxidant capacity of cells, and in doing so help to maintain the health of biological tissues (Xiong, 2010). However, in some situations (e.g. during aging and environmental stress), the efficiency of the endogenous antioxidant diminishes, and the tissues become more vulnerable to free radical attack (Xiong, 2010). Thus, dietary interventions to restore and strengthen the body's antioxidant become necessary. Naturally occurring antioxidant peptides and those derived from protein hydrolysis are now considered as novel and potential dietary ingredients to promote human health. The efficiency of an antioxidant peptide is usually affected by the type of protein substrate, the hydrolytic enzyme used, and the condition (pH, ionic strength, temperature, and preheat treatment) under which peptides are released from a given protein. Many peptides, including those from legumes have been prepared by enzymatic hydrolysis and have been shown to exhibit strong antioxidant activity. Li et al. (2008) demonstrated that four fractions (I, II, III, and IV) from chickpea protein hydrolysates

had strong antioxidant activity as measured using reducing power, inhibition of linoleic acid autoxidation, and 1,1-diphenyl-2-pycrylhydrazyl (DPPH)/superoxide/hydroxyl radical-scavenging assay, with fraction IV having the strongest activity of 81.13%. Humiski and Aluko (2007) carried out antioxidant assay on pea protein hydrolysate with the result showing that the types of enzyme and hydrolysis conditions, as well as amino acid composition and peptide size have strong effects on the antioxidant activity. Generally, there remains insufficient data on role of legumes especially kidney bean proteins and peptides as antioxidant agents. Therefore, there is need for further research in the production and potency evaluation of kidney bean antioxidant peptides.

1.8.2. Blood pressure effects of legumes: Hypertension is a major risk factor for a heart attack and is generally regarded as the greatest risk factor for a stroke. Hypertension is a major, yet controllable, risk factor in cardiovascular disease and related complications. The renin-angiotensin system plays an important role in the development and maintenance of hypertension. Angiotensinogen from the liver produces angiotensin I which is converted by the action of angiotensin-I-converting enzyme (ACE) to the potent vasoconstrictor angiotensin II (Matsui & Tanaka, 2010). The main physiological role of angiotensin II is to exert a vasoconstrictive effect at the vessel wall via the binding of angiotensin II to angiotensin II receptor (AT1) (Millatt, Abdel-Rahman, & Siragy, 1999). Based on this pathway, suppression of angiotensin II production via inhibition of ACE activity will be of great benefit since renin-angiotensin system is involved in ACE action. Small peptides are targeted for developing antihypertensive food products because of their ACE inhibitory action (Matsui & Tanaka, 2010). This is because ACE has four functional amino acid residues of Tyr, Arg, Glu, and Lys at the active site, and three

hydrophobic binding sub-sites which can be blocked by small peptides having high affinity with active sites (Matsui & Tanaka, 2010). It is on this premise that many ACE inhibitory peptides have been developed from natural proteins (Matsui & Matsumoto, 2006). Therapeutic ACE inhibitory drugs such as captopril and enalapril were also designed on the basis of the structure of Ala-Pro or Phe-Ala-Pro (Hooper, 1991). So far, small peptides with hydrophobic and aromatic amino acid residues such as Tyr, Phe, Trp, and Pro at the C-terminal have been shown to have potent ability to inhibit ACE activity with an IC_{50} value of <100 μM (Matsui & Matsumoto, 2006). However, data showing the relationships between kidney bean intakes with blood pressure reduction is scanty.

Renin inhibitors prevent the formation of Angiotensin I from the angiotensinogens and so may act differently from ACE inhibitors which prevent the formation of Angiotensin II from Angiotensin I. A variety of stable peptide-like analogues of the scissile peptide bond of angiotensinogen has been developed and was shown to inhibit renin and lower blood pressure (Weir et al., 2007). Renin inhibitors bind to the active site of renin and inhibit the binding of renin to angiotensinogen which is the rate determining step of the renin-angiotensin-aldosterone system cascade (Brown, 2006). Consequently, renin inhibitors prevent the formation of Angiotensin I and Angiotensin II. Renin inhibitors may also prevent angiotensin 1-7, angiotensin 1-9 and angiotensin 1-5 formation (Müller, Derer, & Dechend, 2008). Renin is highly selective for its only naturally occurring substrate which is angiotensinogen, and the incidence of unwanted side effects with a renin inhibitor is not as common when compared to ACE inhibitors (Weir et al., 2007).

1.9 REFERENCES

Abd El-Hady, E. A., & Habiba, R. A. (2003). Effect of soaking and extrusion conditions on antinutrients and protein digestibility of legume seeds. *LWT - Food Science and Technology, 36*, 285-293.

Agriculture and Agri-food canada. (2009). *Overview of the Canadian pulse Industry;* retrieved from http://www.ats.agr.gc.ca/can/4753-eng.htm on the 04/02/12

Ajibola, C. F., Eleyinmi, A. F., & Aluko, R. E. (2011). Kinetics of the inhibition of renin and angiotensin I converting enzyme by polar and non-polar polyphenolic extracts of vernonia amygdalina and gongronema latifolium leaves. *Plant Foods for Human Nutrition, 66*, 320-327.

Alli, I., Gibbs, B. F., Okoniewska, M. K., Konishi, Y., & Dumas, F. (1993). Identification and characterization of phaseolin polypeptides in a crystalline protein isolated from white kidney beans (*Phaseolus ulgaris*). *Journal of Agricultural and Food Chemistry, 41*, 1830-1834.

Alonso, R., Aguirre, A., & Marzo, F. (2000). Effects of extrusion and traditional processing methods on antinutrients and in vitro digestibility of protein and starch in faba and kidney beans. *Food Chemistry, 68*, 159-165.

Aluko, R. E. (2004). The extraction and purification of proteins: An introduction. In R. Y. Yada (Ed.), *Proteins in food processing* ((pp. 323-346).). Cambridge; Boca Raton: Woodhead Pub; CRC Press.

Amarowicz, R., & Pegg, R. B. (2008). Legumes as a source of natural antioxidants. *European Journal of Lipid Science and Technology, 110*, 865-878.

Anton, A. A., Ross, K. A., Lukow, O. M., Fulcher, R. G., & Arntfield, S. D. (2008). Influence of added bean flour (*Phaseolus vulgaris* L.) on some physical and nutritional properties of wheat flour tortillas. *Food Chemistry, 109*, 33-41.

Annie N.G, Waldron,K.W. (1997). Effect of Cooking and Pre-Cooking on Cell-Wall Chemistry in Relation to Firmness of Carrot Tissues. *Journal of the Science of Food and Agriculture*, 73, 503–512.

Augustin, J., & Klein, B. P. (1989). Nutrient composition of raw, cooked, canned, and sprouted legumes. In Matthews R.H. (Ed.), *Legumes, chemistiy, technology, and human nutrition* (pp. 187-217). New York: Marcel-Dekker.

Berkelman, T., & García-Carreño, F. (2001). Electrophoresis analysis. In V. Chanda (Ed.), *Current protocols in food analytical chemistry* (pp. B3.1.1-B3.1.28) John Wiley & Sons, Inc.

Blat, D., Weiner, L., Youdim, M. B. H., & Fridkin, M. (2008). A novel iron-chelating derivative of the neuroprotective peptide NAPVSIPQ shows superior antioxidant and antineurodegenerative capabilities. *Journal of Medicinal Chemistry, 51*, 126-134.

Bollino, R., & Chrispeels, M. J. (1978). Characterization and subcellular localization of vicilin and phytohemagglutinin, the two major reserve proteins of *Phaseolus vulgaris* L. *Plantachim, 142*, 291-298.

Boniglia, C., Carratù, B., Di Stefano, S., Giammarioli, S., Mosca, M., & Sanzini, E. (2008). Lectins, trypsin and α-amylase inhibitors in dietary supplements containing phaseolus vulgaris. *European Food Research and Technology, 227*, 689-693.

Bonner, P. L. R. (2007). *Protein purification: The basics.* Taylor & Francis.

Boye, J., Zare, F., & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International, 43*, 414-431.

Boye, J. I., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E., & Rajamohamed, S. H. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. *Food Research International, 43*, 537-546.

Brown, M. J. (2006). Direct renin inhibition - A new way of targeting the renin system. *JRAAS - Journal of the Renin-Angiotensin-Aldosterone System, 7*, S7-S11.

Cai, R., Klamczynska, B., & Baik, B. K. (2001). Preparation of bean curds from protein fractions of six legumes. *Journal of Agricultural and Food Chemistry*, 49, 3068−3073.

Carrasco-Castilla, J., Hernández-Álvarez, A. J., Jiménez-Martínez, C., Jacinto-Hernández, C., Alaiz, M., Girón-Calle, J., Dávila-Ortiz, G. (2012). Antioxidant and metal chelating activities of *Phaseolus vulgaris L.* var. jamapa protein isolates, phaseolin and lectin hydrolysates. *Food Chemistry, 131*, 1157-1164.

Chakraborty, P., Sosulski, F. and Bose, A. (1979). Ultracentrifugation of salt-soluble proteins in ten legume species. *Journal of Science and Food Agriculture*. *30*, 766-771.

Chau, C. F., & Cheung, P. C. K. (1998). Functional properties of flours prepared from three chinese indigenous legume seeds. *Food Chemistry, 6*1, 429-433.

Chau, C., Cheung, P. C. K., & Wong, Y. (1997). Functional properties of protein concentrates from three chinese indigenous legume seeds. *Journal of Agricultural and Food Chemistry, 45*, 2500-2503.

Chel-Guerrero, L., PÃrez-Flores, V., Betancur-Ancona, D., & DÃvila-Ortiz, G. (2002). Functional properties of flours and protein isolates from *Phaseolus lunatus* and *Canavalia ensiformis* seeds. *Journal of Agricultural and Food Chemistry, 50*, 584-591.

Chiang, W-D., Tsou, M-J., Tsai, Z-Y., & Tsai, T-C. (2006). Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor. *Food Chemistry, 98*, 725-732.

Clemente, A., Gee, J. M., Johnson, I. T., Mackenzie, D. A., & Domoney, C. (2005). Pea (*pisum sativum L.*) protease inhibitors from the Bowman-Birk class influence the growth of human colorectal adenocarcinoma HT29 cells in vitro *Journal of Agricultural and Food Chemistry, 53*, 8979-8986.

Clementea, A., & Domoney, C. (2006). Biological significance of polymorphism in legume protease inhibitors from the bowman-birk family *Current Protein & Peptide Science, 7*, 201-216.

Coffey, D., Uebersax, M., Hosfield, G., & Bennink, M. (1993). Thermal extrusion and alkali processing of dry beans.*16*421-431.

Coffey, D.G., Uebersax, M.A., Hosfield, G.L. and Brunner, J.R. (1985). Evaluation of the hemagglutinating activity of low-temperature cooked kidney beans. *Journal Food Science*. *50*, 78-81.

Corrêa, A. P. F., Daroit, D. J., Coelho, J., Meira, S. M., Lopes, F. C., Segalin, J., Brandelli, A. (2011). Antioxidant, antihypertensive and antimicrobial properties of ovine milk caseinate hydrolyzed with a microbial protease. *Journal of the Science of Food and Agriculture, 91*, 2247-2254.

Deshpande, S.S., Rangnekar, P.D., Sathe, S.K. and Salunkhe, D.K. (1983). Functional properties of wheat-bean composite flours. *Journal of Food Science 48*, 1659-1662.

Deutscher, M. P. (Ed.). (1990). *Guide to protein purification: methods in enzymology* (182nd ed.). San Diego, California: Academic Press, Inc.

DiLollo, A., Alli, I., Biliarderis, C., & Barthakur, N. (1993). Thermal and surface active properties of citric acid-extracted and alkali-extracted proteins from phaseolus beans. *Journal of Agricultural and Food Chemistry, 41*, 24-29.

Domoney, C., Welham, T., & Sidebottom, C. (1993). Purification and characterization of pisum seed trypsin inhibitors. *Journal of Experimental Botany, 44*, 701-709.

Doonan , S. (2004). Bulk purification by fractional precipitation.. In P. Cutler (Ed.), *Protein purification protocols.methods in molecular biology,* (Second Edition ed., pp. 135-144). Totowa, NJ: Humana Press Inc.

Duranti, M. (2006). Grain legume proteins and nutraceutical properties. *Fitoterapia, 77*, 67-82.

Duranti, M., Lovati, M. R., Dani, V., Barbiroli, A., Scarafoni, A., Castiglioni, S., Morazzoni, P. (2004). The alpha' subunit from soybean 7S globulin lowers plasma lipids and upregulates liver beta-VLDL receptors in rats fed a hypercholesterolemic diet *The Journal of Nutrition, 134*, 1334-1339.

Englard, S., & Seifter, S. (1990). Precipitation techniques. *Methods in Enzymology, 182*, 285-300.

Eskin, N. A. M., & Tamir, S. (2006). *Dictionary of nutraceuticals and functional foods*. Boca Raton, FL: Taylor & Francis Group/CRC Press.

Fernandez-Quintela, A., Macarulla, M. T., del Barrio, A. S., & Martinez, J. A. (1997). Composition and functional properties of protein isolates obtained from commercial legumes grown in northern spain. *Plant Foods Human. Nutrition, 51*, 331-341.

Food and Drug Administration. (1999). Federal register *64*, 57699-57733.

Flink, J. and Christiansen, I. (1973). The production of a protein isolate from *Vicia faba*. *Lebensm Wiss Technology 6*, 102-106.

Fuhrmeister, H., & Meuser, F. (2003). Impact of processing on functional properties of protein products from wrinkled peas. *Journal of Food Engineering, 56*, 119-129.

Fung, W-Y., & Liong, M-T. (2010). Evaluation of proteolytic and ACE-inhibitory activity of lactobacillus acidophilus in soy whey growth medium via response surface methodology. *LWT - Food Science and Technology, 43*, 563-567.

Gómez, M., Oliete, B., Rosell, C. M., Pando, V., & Fernández, E. (2008). Studies on cake quality made of wheat-chickpea flour blends. *LWT - Food Science and Technology, 41*, 1701-1709.

Gonzalez-Gonzalez, C. R., Tuohy, K. M., & Jauregi, P. (2011). Production of angiotensin-I-converting enzyme (ACE) inhibitory activity in milk fermented with probiotic strains: Effects of calcium, pH and peptides on the ACE-inhibitory activity. *International Dairy Journal, 21*, 615-622.

Gueguen, J., & Cerletti, P. (1994). Proteins of some legume seeds: Soybean, pea, fababean and lupin. In B. J. F. Hudson (Ed.), *New and developing sources of food proteins* (pp. 145-183). London: Chapman and Hall.

Han, J., Janz, J., & Gerlat, M. (2010). Development of gluten-free cracker snacks using pulse flours and fractions. *Food Research International, 43*, 627-633.

Harnedy, P. A., & FitzGerald, R. J. (2011). Bioactive peptides from marine processing waste and shellfish: A review. *Journal of Functional Foods,* 4, 6–24.

Hartmann, R., & Meisel, H. (2007). Food-derived peptides with biological activity: From research to food applications. *Current Opinion in Biotechnology, 18*, 163-169.

Hood-Niefer, S. D., & Tyler, R. T. (2010). Effect of protein, moisture content and barrel temperature on the physicochemical characteristics of pea flour extrudates. *Food Research International, 43*, 659-663.

Hooper, N. M. (1991). Angiotensin converting enzyme: Implications from molecular biology for its physiological functions. *International Journal of Biochemistry, 23*, 641-647.

Horisberger, M., & Tacchini Vonlanthen, M. (1983). Ultrastructural localization of Bowman-Birk inhibitor on thin sections of *Glycine* max (soybean) cv. maple arrow by the gold method. *Histochemistry, 77*, 313-321.

Hu, F. B. (2003). Plant-based foods and prevention of cardiovascular disease: An overview. *American Journal of Clinical Nutrition, 78*, 544S-551S.

Humiski, L.M. and Aluko, R.E. (2007). Physicochemical and bitterness properties of enzymatic pea protein hydrolysates. *Journal of Food Science. 72*, S605-S611

Ishino, K., & Ortega, M. L. (1975). Fractionation and characterization of major reserve proteins from seeds of phaseolus vulgaris. *Journal Agricultural and Food Chemistry, 2*3, 529-533.

Jacobs, D. R., & Gallaher, D. D. (2004). Whole grain intake and cardiovascular disease: A review. *Current Atherosclerisis Rep, 6*, 415-423.

Jaffé, W. G., & Hannig, K. (1965). Fractionation of proteins from kidney beans (phaseolus vulgaris). *Archives of Biochemistry and Biophysics, 109*, 80-91.

Jivotovskaya, A. V., Senyuk, V. I., Rotari, V. I., Horstmann, C., & Vaintraub, I. A. (1996). Proteolysis of phaseolin in relation to its structure. *Journal of Agricultural and Food Chemistry, 44*, 3768-3772.

Joubert, F. J. (1957). Ultracentrifuge studies on seed proteins of the family leguminosae. *Journal South African Chemistry Institute, 10*, 16-20.

Jourdan, G. A., Norea, C. P. Z., & Brandelli, A. (2007). Inactivation of trypsin inhibitor activity from brazilian varieties of beans (*Phaseolus vulgaris L.*). *Food Science and Technology International, 13*, 195-198.

Katta, V., & Chait, B. T. (1991). Observation of the heme-globin complex in native myoglobin by electrospray-ionization mass spectrometry. *Journal of the American Chemical Society, 113*, 8534-8535.

Kayitesi, E., De Kock, H. L., Minnaar, A., & Duodu, K. G. (2012). Nutritional quality and antioxidant activity of marama-sorghum composite flours and porridges. *Food Chemistry, 131*, 837-842.

Kelly, S. M., & Price, N. C. (2006). Overview of protein folding. In G. Taylor (Ed.), *Current Protocols in Protein Science* (pp. 20.10.1-20.10.18) John Wiley & Sons, Inc.

Kimura, A. (2008). Comparison of physicochemical properties of 7S and 11S globulins from pea, fava bean, cowpea, and french bean with those of soybean--french bean 7S globulin exhibits excellent properties. *Journal of Agricultural and Food Chemistry, 56*, 10273.

Kinsella, J. E. (1979). Functional properties of soy proteins . *Journal of American oil Chemists Society, 56*, 242-258.

Kiosseoglou, V., & Paraskevopoulou, A. (2011). Functional and physicochemical properties of pulse proteins. In B. K. Tiwari, A. Gowen & B. McKenna (Eds.), *Pulse foods processing,quality and nutraceutical applications.* (First edition ed., pp. 56-90). London: Elsevier.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. applications of bioprocesses used in isolation and recovery *Current Pharmaceutical Design, 9*, 1309-1323.

Kodera, T., & Nio, N. (2006). Identification of an anqiotensin I-converting enzyme inhibitory peptides from protein hydrolysates by a soybean protease and the antihypertensive effects of hydrolysates in spontaneously hypertensive model rats. *Journal of Food Science, 71*, C164-C173.

Koenig, R. L., Singh, S. P., & Gepts, P. (1990). Novel phaseolin types in wild and cultivated common bean (*Phaseolus vulgaris, fabaceae*). *Economic Botany, 44*, 50-60.

Krupa, U., Soral-Śmietana, M., & Lewandowicz, G. (2007). Bean starch – chemical and structural changes upon microwave heat-moisture treatment. In P. Tomasik, V. P. Yuryev & E. Bertoft (Eds.), *Starch: progress in basic and applied science* (pp. 243-254). Warsaw: Polish Society of Food Technologists.

Kutoš, T., Golob, T., Kač, M., & Plestenjak, A. (2003). Dietary fibre content of dry and processed beans. *Food Chemistry, 80*, 231-235.

Lei, H. Y., & Chang, C. P. (2009). Lectin of concanavalin A as an anti-hepatoma therapeutic agent *Journal of Biomedical Science, 16*, 10.

Leiner, I. E. (1975). Effects of anti-nutritional and toxic factors on the quality and utilization of legume. In M. Friedman (Ed.), *1. Protein Nutritional Quality of Foods and Feeds, part 2.* (pp. 523-550). New York: Marcel Dekkar Inc.

Linden, O., Tennvall, J., Hindorf, C., Cavallin-Stahl, E., Lindner, K., Ohlsson, T., Strand, S. (2002). 131I-labelled anti-CD22 MAb (LL2) in patients with B-cell lymphomas failing chemotherapy. treatment outcome, haematological toxicity and bone marrow absorbed dose estimates. *Acta Oncologica, 41*, 297-303.

Lovati, M. R., Manzoni, C., Gianazza, E., Arnoldi, A., Kurowska, E., Carroll, K. K., & Sirtori, C. R. (2000). Soy protein peptides regulate cholesterol homeostasis in hep G2 cells *The Journal of Nutrition, 130*, 2543-2549.

Lovati, M. R., Manzoni, C., Gianazza, E., & Sirtori, C. R. (1998). Soybean protein products as regulators of liver low-density lipoprotein receptors. I. identification of active β-conglycinin subunits. *Journal of Agricultural and Food Chemistry, 46*, 2474-2480.

Manzoni, C., Duranti, M., Eberini, I., Scharnag, H., März, W., Castiglioni, S., & Lovati, M. R. (2003). Subcellular localization of soybean 7S globulin in HepG2 cells and LDL receptor up-regulation by its α′ constituent subunit. *Journal of Nutrition, 133*, 2149-2155.

Martin, C., Maria, A., Waldron, K. W., Maina, G., Grant, G., Bardocz, S. (1995). Hard-to-cook phenomenon in beans*:* Changes in antinutrient factors and nitrogenous compounds during storage. *Journal of the Science of Food and Agriculture, 69*, 429-435.

Matsui, T., & Matsumoto, K. (2006). Antihypertensive peptides from natural resources. In T. H. M. Khan, & A. Ather (Eds.), *Lead molecules from natural products: Discovery and new trends* (pp. 255-271). Amsterdam: Elsevier.

Matsui, T., & Tanaka, M. (2010). Antihypertensive peptides and their underlying mechanisms. In Y. Mine (Ed.), *Bioactive proteins and peptides as functional foods and nutraceuticals* (1st ed., pp. 43-53) Wiley-Blackwell,.

McCarty, M. F. (2005). Nutraceutical resources for diabetes prevention - an update. *Medical Hypotheses, 64*, 151-158.

Mccurdy, S.M. and Kripfel, J.E. (1990). Investigation of faba bean protein recovery and application to pilot scale processing. *Journal Food Science, 55*, 1093-1094.

Medina-Godoy, S., Ambriz-Pérez, D. L., Fuentes-Gutiérrez, C. I., Germán-Báez, L. J., Gutiérrez-Dorado, R., Reyes-Moreno, C., & Valdez-Ortiz, A. (2012). Angiotensin-converting enzyme inhibitory and antioxidative activities and functional characterization of protein hydrolysates of hard-to-cook chickpeas. *Journal of the Science of Food and Agriculture, 92,* 1974-1981.

Mélo, E. A., Stamford, T. L. M., Silva, M. P. C., Krieger, N., & Stamford, N. P. (2003). Functional properties of yam bean (*Pachyrhizus erosus)* starch. *Bioresource Technology, 89*, 103-106.

Mendoza, E. M. T., Adachi, M., Bernardo, A. E. N., & Utsumi, S. (2001). Mungbean [vigna radiata (L.) wilczek] globulins purification and characterization. *Journal of Agricultural and Food Chemistry, 49*(3), 1552-1558.

Mikkonen, A. (1992). Purification and characterization of leucine aminopeptidase from kidney bean cotyledons. *Physiologia Plantarum, 84*, 393-398.

Millatt, L. J., Abdel-Rahman, E. M., & Siragy, H. M. (1999). Angiotensin II and nitric oxide: A question of balance. *Regulatory Peptides, 81*, 1-10.

Mine, Y., Li-Chan, E. C. Y., & Jiang, B. (2010). Biologically active food proteins and peptides in health: An overview. In Y. Mine, E. C. Y. Li - Chan & B. Jiang (Eds.), *Bioactive proteins and peptides as functional foods and nutraceuticals* (1st Edition ed., pp. 5-12) Blackwell Publishing Ltd. and Institute of Food Technologists.

Möller, N. P., Scholz-Ahrens, K. E., Roos, N., & Schrezenmeir, J. (2008). Bioactive peptides and proteins from foods: Indication for health effects. *European Journal of Nutrition, 47*, 171-182.

Moreno, J., Altabella, T., & Chrispeels, M. J. (1990). Characterization of alpha-amylase-inhibitor, a lectin-like protein in the seeds of *Phaseolus vulgaris. Plant Physiology, 92*, 703-709.

Müller, D. N., Derer, W., & Dechend, R. (2008). Aliskiren - mode of action and preclinical data. *Journal of Molecular Medicine, 86*, 659-662.

Chen, H-M., Muramoto, K., Yamauchi, F., & Nokihara, K. (1996). Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. *Journal of Agricultural and Food Chemistry, 44*, 2619-2623.

Nakahara, T., Sano, A., Yamaguchi, H., Sugimoto, K., Chikata, H., Kinoshita, E., & Uchida, R. (2010). Erratum: Antihypertensive effect of peptide-enriched soy sauce-like seasoning and identification of its angiotensin I-converting enzyme inhibitory substances. *Journal of Agricultural and Food Chemistry, 58*, 5858.

Nakahara, T., Sugimoto, K., Sano, A., Yamaguchi, H., Katayama, H., & Uchida, R. (2011). Antihypertensive mechanism of a peptide-enriched soy sauce-like seasoning: The active constituents and its suppressive effect on renin-angiotensin-aldosterone system. *Journal of Food Science, 76*, H201-H206.

Nazeer, R. A., & Srividhya, T. S. (2011). Antioxidant peptides from the protein hydrolysates of *Conus betulinus. International Journal of Peptide Research and Therapeutics, 17*, 231-237.

Nielsen, S. (1991). Digestibility of legume proteins. *Food Technology, 45*(9), 112.

Obiro, W. C., Zhang, T., & Jiang, B. (2008). The nutraceutical role of the *Phaseolus vulgaris* alpha-amylase inhibitor. *British Journal of Nutrition, 100*, 1-12.

Ologhobo, A.D., Apata, D.F., Oyejide, A. and Akinpelu, O. (1993). Toxicity of raw limabeans [Phaseolus Lunatus L.) and limabean fractions for growing chicks. *British Poultry Science 34*, 505-522.

Oomah, B. D., Blanchard, C., & Balasubramanian, P. (2008). Phytic acid, phytase, minerals, and antioxidant activity in canadian dry bean (*phaseolus vulgaris L.*) cultivars. *Journal and Agricultural and Food Chemistry, 56*, 11312-11319.

Osborne, T. B. (1894). The proteids of the kidney bean. *Journal of the American Chemical Society, 16*, 633-643.

Osorio-Díaz, P., Bello-Pérez, L. A., Sáyago-Ayerdi, S. G., Benítez-Reyes, M. D. P., Tovar, J., & Paredes-López, O. (2003). Effect of processing and storage time on in vitro digestibility and resistant starch content of two bean (*phaseolus vulgaris L*) varieties. *Journal of the Science of Food and Agriculture, 83*, 1283-1288.

Otte, J., Lenhard, T., Flambard, B., & Sørensen, K. I. (2011). Influence of fermentation temperature and autolysis on ACE-inhibitory activity and peptide profiles of milk fermented by selected strains of *Lactobacillus helveticus and lactococcus lactis*. *International Dairy Journal, 21*, 229-238.

Paaren, H. E., Slightom, J. L., Hall, T. C., Inglist, A. S., & Blagrove, R. J. (1987). Purification of a seed glycoprotein: N-terminal and deglycosylation analysis of phaseolin. *Phytochemistry, 26*, 335-343.

Pain, R. H. (2003). Determining the CD spectrum of a protein. In Ronald E. W., Terry E. A., E. A. Decker, M. H. Penner, D. S. Reid, S. J. Schwartz, P. Sporns (Eds.), *Current Protocols in Food Analytical Chemistry* (pp. 3.5.1-3.5.25) John Wiley & Sons, Inc.

Pan, D., & Guo, Y. (2010). Optimization of sour milk fermentation for the production of ACE-inhibitory peptides and purification of a novel peptide from whey protein hydrolysate. *International Dairy Journal, 20*, 472-479.

Peñta-Ramos, E. A., & Xiong, Y. L. (2002). Antioxidant activity of soy protein hydrolysates in a liposomal system. *Journal of Food Science, 67*, 2952-2956.

Petitot, M., Boyer, L., Minier, C., & Micard, V. (2010). Fortification of pasta with split pea and faba bean flours: Pasta processing and quality evaluation. *Food Research International, 43*, 634-641.

Pryme, I. F., Bardocz, S., Pusztai, A., & Ewen, S. W. (2006). Suppression of growth of tumour cell lines in vitro and tumours in vivo by mistletoe lectins *Histology and Histopathology, 21*, 285-299.

Pusztai, A., & Watt, W. B. (1970). Glycoprotein II. the isolation and characterization of a major antigenic and non-haemagglutinating glycoprotein from *Phaseolus vulgaris*. *Biochimica et Biophysica Acta, 207*, 413-431.

Qian, B., Xing, M., Cui, L., Deng, Y., Xu, Y., Huang, M., & Zhang, S. (2011). Antioxidant, antihypertensive, and immunomodulatory activities of peptide fractions from fermented skim milk with *Lactobacillus delbrueckii ssp. bulgaricus LB340*. *Journal of Dairy Research, 78*, 72-79.

Habiba, R.A. (2002). Changes in anti-nutrients, protein solubility, digestibility, and HCl-extractability of ash and phosphorus in vegetable peas as affected by cooking methods. *Food Chemistry, 77*, 187-192.

Rangel, A., Domont, G. B., Pedrosa, C., & Ferreira, S. (2003). Functional properties of purified vicilins from cowpea (*Vigna unguiculata)* and pea (*Pisum sativum*) and cowpea protein isolate. *Journal of Agricultural and Food Chemistry, 51*, 5792-5797.

Ruiz-Ruiz, J., Dávila-Ortíz, G., Chel-Guerrero, L., & Betancur-Ancona, D. (2012). Angiotensin I-Converting Enzyme Inhibitory And Antioxidant Peptide Fractions From Hard-To-Cook Bean Enzymatic Hydrolysates. *Journal of Food Biochemistry.*

Ryan, E., Galvin, K., O'Connor, T. P., Maguire, A. R., & O'Brien, N. M. (2007). Phytosterol, squalene, tocopherol content and fatty acid profile of selected seeds, grains, and legumes. *Plant Foods for Human Nutrition, 62*, 85-91.

Sabanis, D., Makri, E., & Doxastakis, G. (2006). Effect of durum flour enrichment with chickpea flour on the characteristics of dough and lasagne. *Journal of the Science of Food and Agriculture, 86*, 1938-1944.

Sai-Ut, S., Ketnawa, P., & Rawdkuen, S. (2009). Biochemical and functional properties of proteins from red kidney, navy and adzuki beans. *Asian Journal of Food and Agro-Industry, 2*, 493-504.

Sánchez-Vioque, R., Clemente, A., Vioque, J., Bautista, J., & Millán, F. (1999). Protein isolates from chickpea (*Cicer arietinum L.*): Chemical composition, functional properties and protein characterization. *Food Chemistry, 64*, 237-243.

Sathe, S. K. (2002). Dry bean protein functionality. *Critical Reviews in Biotechnology, 22*, 175-223.

Schuler, M., Doyle, J., & Beachy, R. (1983). Nucleotide homologies between the glycosylated seed storage proteins of Glycine max and *Phaseolus vulgaris*.*2*(3), 119-127.

Shi, J., Xue, S. J., Kakuda, Y., Ilic, S., & Kim, D. (2007). Isolation and characterization of lectins from kidney beans (*Phaseolus vulgaris*). *Process Biochemistry, 42*, 1436-1442.

Shimelis, E. A., & Rakshit, S. K. (2007). Effect of processing on antinutrients and in vitro protein digestibility of kidney bean (*Phaseolus vulgaris L.*) varieties grown in East Africa. *Food Chemistry, 103*, 161-172.

Siddiq, M., Butt, M. S., & Sultan, M. T. (2011). Dry beans: Production, processing, and nutrition. In N. K. Sinha (Ed.), *Handbook of vegetables and vegetable processing* (pp. 545-564). Ames, Iowa: Wiley-Blackwell.

Sikorski, Z. E. (Ed.). (2006). *Chemical and functional properties of food components* (Third Edition ed.) CRC Press.

Sirtori, C. R., Galli, C., Anderson, J. W., & Arnoldi, A. (2009). Nutritional and nutraceutical approaches to dyslipidemia and atherosclerosis prevention: Focus on dietary proteins. *Atherosclerosis, 203*, 8-17.

Sparvoli, F., Faoro, F., Daminati, M. G., Ceriotti, A., & Bollini, R. (2000). Misfolding and aggregation of vacuolar glycoproteins in plant cells. *Plant Journal, 24*, 825-836.

Stadtman, E. R. (2001). *Protein oxidation in aging and age-related diseases.* Annals of the New York Academy of Sciences, 928. 22-38.

Sturm, A., Van Kuik, J. A., Vliegenthart, J. F., & Chrispeels, M. J. (1987). Structure, position, and biosynthesis of the high mannose and the complex oligosaccharide side chains of the bean storage protein phaseolin. *Journal of Biological Chemistry, 262*, 13392-13403.

Sun, S. M., & Hall, T. C. (1975). Solubility characteristics of globulins from *phaseolus* seeds in regard to their isolation and characterization. *Journal of Agricultural and Food Chemistry, 23*, 184-189.

Sun, S. M., McLeester, R. C., Bliss, F. A., & Hall, T. C. (1974). Reversible and irreversible dissociation of globulins from *Phaseolus vulgaris* seed. *Journal Biological Chemistry, 249*, 2118-2121.

Tang, C-H. (2008). Thermal denaturation and gelation of vicilin-rich protein isolates from three *Phaseolus* legumes: A comparative study. *LWT - Food Science and Technology, 41*, 1380-1388.

Tang, C-H. & Ma, C-Y (2009). Heat-induced modifications in the functional and structural properties of vicilin-rich protein isolate from kidney (*Phaseolus vulgaris L*) bean. *Food Chemistry, 115*, 859-866.

Tang, C-H., Sun, X., Yin, S., & Ma, C-H. (2008). Transglutaminase-induced cross-linking of vicilin-rich kidney protein isolate: Influence on the functional properties and in vitro digestibility. *Food Research International, 41*, 941-947.

Tharanathan, R. N., & Mahadevamma, S. (2003). Grain legumes—a boon to human nutrition. *Trends in Food Science & Technology, 14*, 507-518.

Tyler, R. T. (1984). Impact milling quality of grain legumes. *Journal of Food Science, 49*(3), 925-930.

Uebersax, M. A., Ruengsakulrach, S., & Occena, L. (1991). Strategies and procedures for processing dry bean. *Food Technology, 45*, 104-108.

Vaintraub, I. A., Bassüner, R., & Shutov, A. D. (1976). The action of trypsin and chymotrypsin on the reserve proteins of some leguminous seeds. *Food / Nahrung, 20*(8-9), 763-771.

Valdez-Ortiz, A., Fuentes-Gutiérrez, C. I., Germán-Báez, L. J., Gutiérrez-Dorado, R., & Medina-Godoy, S. (2012). Protein hydrolysates obtained from azufrado (sulphur yellow) beans (*Phaseolus vulgaris)*: Nutritional, ACE-inhibitory and antioxidative characterization. *LWT - Food Science and Technology, 46*, 91-96.

van Heerden, S. M., & Schönfeldt, H. C. (2004). The need for food composition tables for southern africa. *Journal of Food Composition and Analysis, 17*, 531-537.

Vidal-Valverde, C., Frias, J., Diaz-Pollan, C., Fernandez, M., Lopez-Jurado, M., & Urbano, G. (1997). Influence of processing on trypsin inhibitor activity of faba beans and its physiological effect. *Journal of Agricultural and Food Chemistry, 45*(9), 3559-3564.

Vose, J. (1980). Production and functionality of starches and protein isolates from legume seeds (field peas and horsebeans). *Cereal chemistry 57*, 406-410.

Wang, Z-L., Zhang, S-S., Wang, W., Feng, F-Q. & Shan, W-G. (2011). A novel angiotensin I converting enzyme inhibitory peptide from the milk casein: Virtual screening and docking studies. *Agricultural Sciences in China, 10*, 463-467.

Wan, X.S., Serota, D.G., Ware, J.H., Crowell, J.A., Kennedy, A.R., (2002). Detection of Bowman-Birk inhibitor antibodies in sera of humans and animals treated with Bowman-Birk inhibitor concentrate. *Nutrition and Cancer* 43, 167–173.

Waterman, H. C., Johns, C. O., & Jones, D. B. (1923). Conphaseolin: A new globulin from the navy bean, *Phaseolus vulgaris*. *Journal Biological Chemistry, 55*, 93-104.

Weir, M. R., Bush, C., Anderson, D. R., Zhang, J., Keefe, D., & Satlin, A. (2007). Antihypertensive efficacy, safety, and tolerability of the oral direct renin inhibitor aliskiren in patients with hypertension: A pooled analysis. *Journal of the American Society of Hypertension, 1*, 264-277.

Wood, J. A., & Malcolmson, L. J. (2011). Substitution of pulse flours into other products. In B. K. Tiwari, A. Gowen & B. McKenna (Eds.), *Pulse foods processing, quality and nutraceutical applications* (First edition ed., pp. 214). London: Elsevier.

Wood, J. A. (2009). Texture, processing and organoleptic properties of chickpea-fortified spaghetti with insights to the underlying mechanisms of traditional durum pasta quality. *Journal of Cereal Science, 49*, 128-133.

Wu, J., Aluko, R. E., & Muir, A. D. (2009). Production of angiotensin I-converting enzyme inhibitory peptides from defatted canola meal. *Bioresource Technology, 100*, 5283-5287.

Xiong, Y. L. (2010.). Antioxidant peptides. In Y. Mine (Ed.), *Bioactive proteins and peptides as functional foods and nutraceuticals* (1st Edition ed., pp. 29-39) Wiley-Blackwell.

Xu, B. J., & Chang, S. K. C. (2008). Total phenolic content and antioxidant properties of eclipse black beans (*Phaseolus vulgaris L.*) as affected by processing methods. *Journal of Food Science, 73*, 19-27.

Yach, D., Hawkes, C., Gould, C.L. and Hofman, K.J. (2004). The global burden of chronic diseases: Overcoming impediments to prevention and control. *Journal American Medicine Association 291*, 2616-2622.

Yeo, S.K.., & Liong, M.T. (2010). Angiotensin I-converting enzyme inhibitory activity and bioconversion of isoflavones by probiotics in soymilk supplemented with prebiotics. *International Journal of Food Sciences and Nutrition, 61*, 161-181.

Yin, SW., Tang, C-H., Wen, Q-B., Yang, X-Q., & Li, L. (2008). Functional properties and in vitro trypsin digestibility of red kidney bean (*Phaseolus vulgaris L.)* protein isolate: Effect of high-pressure treatment. *Food Chemistry, 110*, 938-945.

Yu, Z., Liu, B., Zhao, W., Yin, Y., Liu, J., & Chen, F. (2012). Primary and secondary structure of novel ACE-inhibitory peptides from egg white protein. *Food Chemistry,*

Zhang, T., Jiang, B., Mu, W. and Wang, Z. (2009). Emulsifying properties of chickpea protein isolates: Influence of pH and NaCl. *Food Hydrocolloids 23,* 146-152.

Zhang, T., Li, Y., Miao, M., & Jiang, B. (2011). Purification and characterisation of a new antioxidant peptide from chickpea (*Cicer arietium L.*) protein hydrolysates. *Food Chemistry, 128*, 28-33.

Zhou, D-Y., Tang, Y., Zhu, B-W., Qin, L., Li, D-M., Yang, J-F. &., Murata, Y. (2012). Antioxidant activity of hydrolysates obtained from scallop (*Patinopecten yessoensis*) and abalone (*Haliotis discus hannai ino*) muscle. *Food Chemistry, 132*, 815-822.

Zhu, X., Watanabe, K., Shiraishi, K., Ueki, T., Noda, Y., Matsui, T., & Matsumoto, K. (2008). Identification of ACE-inhibitory peptides in salt-free soy sauce that are transportable across caco-2 cell monolayers. *Peptides, 29*, 338-344.

CHAPTER 2

PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES OF KIDNEY BEAN ALBUMIN AND GLOBULIN PROTEIN FRACTIONS

S. Mundi, R.E. Aluko*

The Department of Human Nutritional Sciences and the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

*Corresponding author: Rotimi E. Aluko, Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, MB R3T 2N2, Canada; E-mail: alukor@cc.umanitoba.ca, Tel: +1(204) 474-9555, Fax: +1(204) 474-7593

1.0. **Abstract**

Albumin and globulin proteins obtained after ammonium sulfate precipitation of proteins from red kidney beans were investigated for their functional properties. Protein contents of the fractions were found to be 40 and 91.5% for albumin and globulin, respectively. Amino acid analysis showed that sulfur-containing amino acids (cysteine and methionine) were limiting but the proteins were rich in acidic amino acids. SDS-PAGE pattern of the globulin fraction revealed one major polypeptide (7S vicilin) with molecular weight (MW) of ~45 kDa and minor 11S legumin polypeptides (MW >90 but <150 kDa) in addition to other minor low MW polypeptides. The solubility profiles showed minimum values at pH 4-6 for globulin but the albumin was more than 60% soluble at pH 3-9. Water holding and oil binding capacity were, respectively, 3.4 and 2.37 mL/g for albumin, which were significantly higher ($p<0.05$) than the values of 2.56 and 1.87mL/g for globulin. The least gelation concentration was 6% for globulin compared with a significantly higher ($p<0.05$) 16% for albumin.

Keywords: Kidney beans; Albumin; Globulin; Functional properties; physicochemical properties

2.1. Introduction

Red kidney bean (*Phaseolus vulgaris*) is a key food source for humans because the seed is low in fat and it is a very good source of folate and fibre (Shi, Xue, Kakuda, Ilic, & Kim, 2007). For many people in the world, the seed is an important and economical source of high dietary protein (Shi et al., 2007). This is because kidney beans contain up to 20-30% protein on a dry weight basis, and have been shown to have good amino acid composition (Yin, Tang, Wen, Yang, & Li, 2008), which makes the seed a good candidate for the formulation of nutritious foods. However, kidney beans contain substantial amounts of lectins which have both beneficial and harmful biological properties. Lectins which are carbohydrate binding proteins found in kidney bean are considered antinutrients factors because of their resistance to digestive enzymes as well as their partial resistance to thermal denaturation (Shi et al., 2007). Because of these anti-nutritional factors, additional processing steps such as cooking must be included in order to make the beans safe to eat (Shi et al., 2007; Zhang et al., 2008). On the other hand, in addition to contributions to human nutrition, a variety of beneficial biological activities such as antitumor, antihistamine, immunomodulatory, antifungal, anti-human immunodeficiency virus (HIV) and anti-insect activities have been reported for kidney bean proteins (Eskin, & Tamir, 2006).

Given the reported health effects and potential therapeutic properties of kidney beans, data derived from studies of the functional and nutritional properties may enhance industrial utilization. This is because the structure-function relationships of plant proteins affect their behavior in food systems during preparation, processing, storage and consumption. The physicochemical and functional properties of legume flours, protein

isolates and their 7S globulins have been studied, with results demonstrating good functional properties (Chau & Cheung, 1998; Njintang, Mbofung, & Waldron, 2001). Kidney bean flours for example, had higher gelation capacities than soybean flour and emulsifying activities and foam stability were comparable (Chau & Cheung, 1998; Njintang et al., 2001). Previous findings also showed that the thermal denaturation properties were higher and the least gelation concentration of kidney bean protein isolate was lower than those of mung bean (*Phaseolus aureus*) protein isolates (Tang, 2008). Whereas information is available on protein isolates and globulins (Yin, Tang, Wen, & Yang, 2010; Tang & Ma, 2009; Yin et al., 2008), there is scanty information on functional properties of the albumin fraction from red kidney beans. Albumins and globulins constitute the main proteins in kidney beans. In this study therefore, albumin and globulin fractions, obtained as the soluble component after ammonium sulfate precipitation of other proteins from red kidney beans were compared for their functional properties. Results from this work could provide fundamental information on the structural and functional properties that could facilitate potential application of kidney bean protein fractions in the formulation of new food products.

2.2. Materials and Methods

2.2.1. Materials

The red kidney bean (*Phaseolus vulgaris L.*) seeds used for all experiments were purchased from a local store in Winnipeg, Manitoba. The seeds were cleaned and milled into powder using a Retsch ZM200 centrifuge mill (Retsch GmbH, Haan, Germany). The powder was stored at 4^{o}C until used for protein extraction.

2.2.2. Protein extraction

Kidney bean flour was extracted and fractionated into albumin and globulin using slight modifications of a previously described protocol (Aluko, 2004). The flour was mixed with 0.1 M phosphate buffer, pH 8 (1:10 w/v) for 2 h at 4^{o}C while stirring. The supernatant collected after centrifugation (9000 x *g*, 45 min at 4^{o}C) was mixed at 4^{o}C with solid ammonium sulfate until 40% saturation was reached. After stirring for 2 h, the slurry was centrifuged (9000 x *g*, 45 min at 4^{o}C) and the precipitate discarded. The supernatant was further adjusted to 80% saturation with addition of solid ammonium sulfate, stirred, and centrifuged as before. The precipitate was collected, dispersed in water and then dialyzed against water at 4^{o}C for 5 days using dialysis tubing of 6-8 kDa molecular weight cut off; dialysis water was changed 3 times daily. The contents of the dialysis bag was then centrifuged (9000 x *g*, 45 min at 4^{o}C) to obtain globulin (precipitate) and albumin (supernatant), which were individually freeze dried and used for subsequent analyses. Protein concentration was determined using the modified Lowry method (Markwell, Haas, Biebar, & Tolbert, 1978).

2.2.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The freeze dried globulin and albumin fractions were subjected to SDS-PAGE (reducing and non-reducing) according to the method of Aluko & McIntosh (2004) with minor modifications. The freeze-dried protein was dispersed (approx. 10 mg/mL) in Tris/HCl buffer, pH 8.0 containing SDS or SDS + 10% β-mercaptoethanol, followed by heating at 95^{o}C for 10 min, cooling and centrifugation. After centrifugation, approximately 1 µl of supernatant was loaded onto 8-25% gradient gels and electrophoresis performed with Phastsystem Separation and Development units according to the manufacturer's

instructions (GE Healthcare, Montréal, PQ, Canada). A mixture of standard proteins (10-225 kDa) was used as the molecular weight marker, and the gels were stained with coomassie blue.

2.2.4. Amino acid analysis

An HPLC system was used to determine the amino acid profiles after samples were hydrolyzed with 6 M HCl as previously reported (Bidlingmeyer, Cohen, & Tarvin, 1984). The cysteine and methionine contents were determined after performic acid oxidation (Gehrke, Gehrke, Wall, Absheer, Kaiser, & Zumwalt, 1985) and tryptophan content was determined after alkaline hydrolysis (Landry & Delhaye, 1992).

2.2.5. Protein solubility

Protein solubility of albumin and globulin was determined according to the method of Aluko & Yada (1995). Briefly, at pH 3.0-9.0 values, 1 mg of sample was dispersed in 1 mL of 0.1 M phosphate buffer solutions to obtain a 0.1% (w/v) concentration and the resulting mixture was vortexed for 2 min and centrifuged at 10000 x *g* for 20 min. Protein content of the supernatant was determined using the modified Lowry method (Markwell et al., 1978). Total protein content was determined by dissolving the isolated globulin or albumin in 0.1 M NaOH solution. Protein solubility (PS) was expressed as percentage ratio of supernatant protein content to the total protein content.

2.2.6. Foam formation

Foams were formed as previously described (Aluko et al. 2009) using slurries that were prepared by dispersing 0.1-0.3g samples in 50 mL graduated centrifuge tubes containing 5 mL 0.1 M phosphate buffer, pH 3.0, 5.0, 7.0, and 9.0. Sample slurries were homogenized at 20,000 rpm for 1 min using a 20 mm foaming shaft on the polytron PT

3100 homogenizer (Kinematica AG, Lucerne, Switzerland). The capacity of the continuous phase to include air (foam capacity) using the mean of three measurements was determined thus;

$$\text{Foam Capacity (FC)} = \frac{\text{Volume after homogenization-Volume before homogenization}}{\text{Volume before homogenization}}$$

The ability to retain air for a certain period of time (foam stability) was calculated by measuring the foam volume after storage at room temperature for 30 min and expressed as percentage of the original foam volume.

2.2.7. Emulsion formation and measurement

The method of Aluko et al. (2009) was used to prepare an oil-in-water emulsion with some modifications. Protein slurries of 50, 125, or 250 mg/mL concentrations were prepared in 5 mL of 0.1 M phosphate buffer pH 3.0, 5.0, 7.0, or 9.0 followed by addition of 1 mL of pure canola oil. The oil/water mixture was homogenized at 20,000 rpm for 1 min, using the 20 mm non-foaming shaft on a Polytron PT 3100 homogenizer. The oil droplet size ($d_{3,2}$) of the emulsions was determined using Mastersizer 2000 (Malvern Instruments Ltd., Malvern, U.K.) with distilled water as dispersant. Under constant shearing, an emulsion sample taken from the bottom of the tubes was added to about 100 mL of water contained in the small volume wet sample dispersion unit (Hydro 2000S) attached to the instrument until the required level of obscuration was attained. The instrument was set to automatically measure each emulsion in triplicate and each emulsion was prepared thrice. Emulsions were kept at room temperature for 1 h without agitation and the particle size distribution and mean particle diameter were measured again to assess stability (percentage ratio of oil droplet size at time zero to oil droplet size measured at 1 h).

2.2.8. Total and exposed sulfhydryl contents

Sulfhydryl and total cysteine contents were determined by a modification of the method of Beveridge et al. (1974). For the sulfhydryl group, samples (15 mg each) were suspended in 3 mL of the reaction buffer (Tris-glycine buffer, pH 8.0) as follows; i) without 8 M urea (exposed SH), ii) with 8 M urea (total SH). After addition of 50 µl of the Ellman's reagent, the resultant suspensions were incubated for 1 h at room temperature with occasional shaking and then centrifuged for 25 min at 10,000 x g. The absorbance of the supernatant was measured at 412 nm using the mixture of reagent buffer and Ellman's reagent as the blank. To determine the total cysteine level, 0.5 mL mercaptoethanol was added and incubated for 1 h at 25^{o}C followed by centrifugation for 25 min at 10,000 x g. An aliquot (1 mL) of the supernatant was transferred into a test-tube and 10 mL of 12% trichloroacetic acid (TCA) was added and held for another hour at 25^{o}C. The mixture was centrifuged for 15 min at 10,000 x g and the precipitate was suspended twice in 5 mL TCA, centrifuged each time and then dissolved in 3 mL Tris buffer without urea. The color was developed with 50 µL Ellman's reagent and absorbance read at 412 nm. The sulfhydryl concentration (total and exposed) in µmol/g of protein was calculated by using the extinction coefficient of 2-nitro-5-thiobenzoate (NTB) at 412 nm (13 600 mol L^{-1} cm^{-1}):

$$\mu\text{mol SH/g protein} = \frac{73.53A \times D}{C}$$

Where A = the absorbance at 412 nm; C = the sample concentration in mg solids/mL; D = dilution factor; and 73.53 is derived from $10^6/ (1.36 \times 10^4)$; 1.36×10^4 is the molar absorptivity and 10^6 is for conversions from the molar basis to the µM/mL basis and from mg solids to g solids.

The absorbance at 412 nm for samples without the Ellman's reagent and the absorbance for the Ellman's reagent in the sample buffer were deducted from the absorbance for samples together with the Ellman's reagent.

2.2.9. Water and oil holding capacity

The water and oil holding capacity were determined using the method of Beuchat (1977) with some modifications. Protein sample (3g) was dispersed in 25 mL distilled water (or pure canola oil) in a 50 mL pre-weighed centrifuge tube. The dispersions were vortexed for 1 min, allowed to stand for 30 min and then centrifuged at 7,000 x g for 25 min at room temperature. The supernatant was decanted, excess water (or oil) in the upper phase drained for 15 min and tube containing the protein residue was weighed again to determine amount of water or oil retained per gram of sample.

2.2.10. Least gelation concentration (LGC)

Least gelation concentration was determined according as previously described (Aluko et al., 2009) by suspending the samples in water at different concentrations (2-20%, w/v). The mixtures were vortexed, placed in a water bath at 95^{o}C for 1 hr, cooled under tap water and left in the refrigerator (4^{o}C) for 14 hrs. The sample concentration at which the gel did not slip when the tube was inverted was taken as the LGC.

2.2.11. Total carbohydrate content

Total carbohydrate content was determined as described by Yem & Willis (1954). Standard glucose stock solution was prepared by dissolving 100 mg in 100 mL water, and then 10 mL of stock was further diluted to 100 mL with distilled water. The solution was refrigerated until analysis after adding a few drops of toluene. Sample (100 mg) was weighed into boiling tube and hydrolyzed in a boiling water bath for 3 h with 5 mL of 2.5

M HCl and cooled to room temperature. Solid sodium carbonate was added until the effervescence ceased. The volume was made up to 100 mL and centrifuged; the supernatant (0.5 and 1 mL aliquots) was taken for analysis. A standard curve was prepared by dispensing varied volume of the glucose standard (0, 0.2, 0.4, 0.6, 0.8 and 1 mL) where '0' served as blank. The volume was made up to 1 mL in all the tubes including the sample tubes by adding distilled water. Then 4 mL of anthrone reagent was added and heated for eight minutes in a boiling water bath, cooled rapidly and the green to dark green colour was read at 630 nm. From the plot of concentration of standard versus absorbance, the amount of carbohydrate present in the sample tube was calculated as:

$$\text{Amount of carbohydrate present in 100 mg of the sample} = \frac{\text{mg glucose}}{\text{Sample vol (mL)}} \times 100$$

2.2.12. Statistical analysis

Results were expressed as the mean values ± standard deviation (SD) after subjecting the averages of triplicate determinations to ANOVA and Duncan's multiple range tests using Statistical Analysis Systems (SAS) desktop software, version 9.2. Differences at $p \leq 0.05$ were considered significant.

2.3. Results and Discussion

2.3.1. Amino acids composition

The composition of amino acids expressed as percent contents for the whole flour, albumin and globulin is reported in Table 2.1. The results demonstrate a nutritionally acceptable amino acid composition for kidney bean protein isolate due to the high levels of essential residues such as lysine, isoleucine, leucine, phenylalanine and valine. Amino acids profile found in this study is similar to previously reported data for vicilin-rich protein isolates from three *Phaseolus* legumes (Tang et al., 2008) and for red kidney

beans protein isolates (Hang, Steinkraus, & Hackler, 1980). The ratio of essential to total amino acids is 42.98, 47.56 and 44.64% for whole flour, albumin and globulin respectively. These values are above the 35% upper range for total protein in the diet as a percent of total energy intake (Dietary Reference Intakes (DRI), 2005). This study shows different levels of acidic, basic and sulfur-containing amino acids. As expected, the fractions have low contents of sulfur-containing amino acids (cysteine and methionine), which are the limiting amino acids in legume seed proteins (Wu et al., 1996). The albumin contained 30 and 2 folds higher contents of cysteine and methionine, respectively when compared to globulin. The whole flour and the fractions were found to be high in aspartic acid/asparagine and glutamic acid/glutamine (acidic amino acids), which is typical of seed storage proteins as previously reported (Tang & Ma, 2009). Together, dietary glutamine and asparagine end up in tissues where they serve as important reservoirs of amino groups for the body (Vasconcelos et al., 2010). In addition, glutamine has received attention as an important fuel source for the intestinal tract, especially in the control of glycogen synthesis and protein degradation (Mahan & Escott-Stump, 1996).

2.3.2. Yield and protein solubility (PS)

The gross yields for albumin and globulin were 4.1 and 22.2% (w/w of flour) whereas the protein yields were 7.2 and 88.0% (w/w protein content of flour), respectively for albumin and globulin fractions (Table 2.2). The results confirm that the globulins are the major proteins in kidney bean seeds while albumins represent a minor fraction; in total both protein fractions constitute the major proteins. The pH-dependent PS profile for the albumin and globulin fractions is shown in Fig. 2.1. The PS in

phosphate buffer showed minimum values at around pH 4-6 for globulin, whereas the albumin was soluble at most pH values. The graph for the globulin had minimum solubility at around pH 4.8 near the isoelectric point and increased steadily from pH 4.0 and 6.0 toward the acidic and basic pH values, respectively. Similar observations have been reported for kidney bean protein isolate (Sai-Ut, Ketnawa, & Rawdkuen, 2009; Yin et al., 2010) and cowpea proteins (Ragab, Babiker, & Eltinay, 2004). The significantly higher ($p<0.05$) protein solubility of the albumin could be attributed to the glycoprotein nature (45% carbohydrate content) when compared to globulin (3.9% carbohydrate content) (Table 2.2). Therefore, the high solubility of albumin suggests that the carbohydrate component may have enhanced protein interactions with water. However, the high PS of the albumin at the acidic pH range may also be due to its high isoelectric point and is similar to data reported for buckwheat albumins (Tang & Wang, 2010).

2.3.3. Gel Electrophoresis

It has been shown that variations in polypeptide profiles have consequences, particularly on the emulsion or gelation capacity of proteins, which are important functional properties of protein samples (Aluko & McIntosh, 2001). In this study, SDS-PAGE was used to separate proteins according to their electrophoretic mobility as a function of polypeptide molecular weight. Except for the presence of some small and large MW polypeptide subunits, the electrophoretic patterns for globulin under non-reducing (lane 2) and reducing (lane 3) conditions as shown in Fig. 2.2 revealed two major polypeptides with estimated molecular weights of approximately 43 & 45 kDa, which belongs to the 7S vicilin (Chagas and Santoro, 1997; Sathe, 2002; Shi et al., 2007; Hou, Hou, Yanyan, Qin, & Li, 2010). In addition to the two main polypeptides, there

were three minor polypeptide chains (<30 kDa) for the globulin fraction as evident in lanes 2 & 3 (Fig. 2.2). Addition of mercaptoethanol did not have any substantial effect on the globulin fraction with the exception of reduced intensity of polypeptides that were <15 kDa, suggesting presence of disulfide bonds in these small size polypeptides. The albumin fraction under non-reducing (lane 4) and reducing (lane 5) conditions, in contrast to the globulins, comprised predominantly of protein subunits with sizes <30 kDa, with the major polypeptide band at around 25 kDa which corresponds to lectins, also known as haemagglutinins (Sathe, 2002). Addition of mercaptoethanol also had minimal effect on the albumin polypeptides with the exception of the minor band at <10 kDa, which had reduced intensity.

2.3.4. Oil and water holding capacity

The oil holding capacity (OHC) could influence emulsifying capacity, a highly desirable characteristic of proteins and protein products such as mayonnaise (Sai-Ut et al., 2009). The OHC is also important for the development of new food products with flavor binding properties and ability to reduce rate of oxidative rancidity since high OHC allows the stabilisation of high fat food products (Grigelmo-Miguel, Gorinstein, & MartõÂn-Belloso, 1999). In the current study, oil absorption capacity for albumin and globulin as reported in Table 2.2 are 2.37 and 1.87 mL/g, respectively in comparison to 1.40 mL/g for red bean globulin (Meng & Ma, 2002), 1.7 mL/g for chickpea protein isolate (Lopez et al., 1991) and 1.5 mL/g for sesame protein isolate (Khalida, Babikerb, & EI-Tianay, 2003). It is possible that the reduced OHC for the globulin is due to the more globular protein structure that limits interaction with the lipid phase when compared to the more open structure of the albumins. In comparison to many plant seeds, kidney

bean proteins, especially the albumin fraction, has higher OHC and therefore, when combined with the high water holding capacity discussed below, can be considered in food formulation since oil and water absorption is essential in the formulation of many processed foods (Foh, Amadou, Kamara, Foh, & Wenshui, 2011).

Water Holding Capacity (WHC) is the ability of the protein to hold water against gravity. Kidney bean albumin held 3.4 mL/g as compared to 2.56 mL/g held by the globulin fraction. The WHC values in the current study are within the range reported for other legumes (assuming that 1 mL of water is equivalent to 1 g of water): between 2.65 and 3.80 g/g for flours and protein isolates of lima beans (*Phaseolus lunatus*) (Chel-Guerrero, Pérez-Flores, Betancur-Ancona, & Dávila-Ortiz, 2002), and 3.0 g/g for red beans, 2.9 g/g black beans, and 2.9 g/g for white, as well as mung bean (2.1 g/g) (Dzudie & Hardy, 1996). However, the values are lower than those reported for protein isolates from *P. calcaratus* (5.28 g/g) and *P. angularis* (5.05 g/g) (Chau, Cheung, & Wong, 1997), probably because of the isolates contain more hydrophilic impurities than the albumin and globulin fractions. The higher WHC of the albumin may be attributed to a more open structure and greater flexibility that enhances interaction with water when compared to the more globular structure of globulins. Previous reports have suggested water absorption capacity ranging from 1.49 to 4.72 g/g is recommended for use in viscous foods (Aletor, Oshodi, & Ipinmoroti, 2002). Therefore, the WHC values obtained in the current study suggest that red kidney bean globulin and albumin may be important ingredients during formulation of food products that require high water retention.

2.3.5. Gelation Capacity

The gelation capacity as shown in Table 2.2 indicates higher gel formation capacity for the globulin (LGC of 6%) when compared to albumin (LGC of 16%). The LGC value obtained in this work for red kidney bean globulin is lower than the 10% value reported for defatted cashew nut concentrate (Adebowale & Adebowale, 2007), 14% for soybeans (Okezie & Bello, 1988), 12% for lupin protein concentrate (Lqari, Vioque, Pedroche, & Millan, 2002), and 7.5% for wheat protein isolate (Schmidt, 1981). The better gel-forming ability of the kidney bean globulin may be due to lower levels of impurities when compared to protein concentrates and isolates that have higher impurity levels. The capacity to form a gel under practical conditions is an important functionality in many food proteins. The ability of protein to form gel and provide a structural matrix for holding water, flavours, sugars and food ingredients is useful in food applications (Kinsella, 1979). Since protein-protein interactions are required during gelation, the presence of high levels of hydrophilic sugar moieties encouraging protein-water interactions in the albumin fraction may have contributed to reduced ability to form gels. However, the reduced gelling ability of the albumin may also be due to lower numbers of hydrophobic clusters as previously shown for buckwheat albumin (Tang & Wang, 2010), which could also limit protein-protein interactions.

2.3.6. Sulfhydryl (SH) and total cysteine content

The results of SH and total cysteine contents are presented in Table 2.2. The total (exposed + buried) and exposed SH contents were, respectively, 4.6 and 2.2 μmol/g for albumin, 3.4 and 1.4 μmol/g for globulin, while total cysteine contents for albumin and globulin were 1.12 and 0.052 μmol/g protein, respectively. The high content of SH group could have contributed to increased interaction with water, which enhanced WHC and

protein solubility better than the globulin that had lower SH content. These values are within the range of values published previously for kidney beans isolate (Tang, 2008; Tang & Ma, 2009). The values obtained for albumin are higher, since albumin proteins are generally richer in SH and cysteine content (Genovese & Lajol, 1998). The obtained values for SH groups could be attributable to the non-vicilin components reported earlier to be about 16% in the kidney bean protein isolate, since the major protein (vicilin) lacks cysteine residues (Tang & Ma, 2009). Results of the cysteine contents obtained from the Ellman's reaction were in agreement with values shown in Table 2.1 for the amino acid contents of albumin and globulin.

2.3.7. Foaming Capacity (FC) and Foam stability (FS)

The effects of pH and protein concentration on FC of albumin and globulin proteins of kidney beans are shown in Fig. 2.3. In addition to adsorption at the air/water interface during bubbling, the solubility, conformational change and rearrangement at the interface, as well as cohesive viscoelastic film formation via intermolecular interactions are basic requirements for a protein to be a good foaming agent **(**Sanchez-Vioque, Vioque, Bautista, & Millan, 1999; Bora, 2002**)**. Generally, for albumin and globulin the trends in the variation was pH dependent with the minimum percentage foaming capacity observed at pH 5, which is the point of least protein solubility, and solubility is a prerequisite for good foaming properties. Ragab et al. (2004) and Lawal et al. (2005) also observed pH-dependent foaming characteristics in cowpea protein isolate and foam capacities of albumin and globulin of African locust bean, respectively. For the albumin and globulin proteins, foaming capacity increased significantly ($p \leq 0.05$) with increasing sample concentration at all pH values, though the globulin had significantly less ($p < 0.05$)

FC (76%) when compared to albumin (100%). The lower FC of globulin proteins may be due to reduced ability of the globular proteins to unfold at the air-water interface, which limits capacity to encapsulate air bubbles (Sai-Ut et al., 2009). In contrast, the albumin was very soluble in aqueous buffer and the increased interaction with water would have enhanced protein unfolding and ability to enhance the formation of air bubbles. Specific properties of protein films such as film thickness and mechanical strength are required for the foam to be stable. In addition, foam stability requires the formation of cohesive, viscous, elastic, continuous, air-impermeable film around each gas bubble, and pH and temperature are crucial factors to foam stability (Sai-Ut et al., 2009). Therefore, the higher foam stability found in the globulin fraction compared to the albumin, as shown in Fig. 2.4 suggests that the higher protein content enhances greater protein-protein interactions to form strong interfacial membranes at the air-water interface. This is probably due to the high level of sugars in the albumin coupled with the low surface hydrophobicity, which would reduce protein-protein interactions and lead to formation of weak interfacial membranes that are unable to stabilize the foams for extended periods of time.

2.3.8. Emulsion Capacity and Stability

As shown in Fig. 2.5, the surface diameter ($d_{3,2}$) showed consistent droplet size reduction, especially for albumin, and in some cases for globulin, at most pH as sample concentrations increased from 50 to 250 mg/mL, which indicates that increase in protein content may have beneficial effect on quality of oil-in-water emulsions. This trend has been reported previously for pea seed flour fractions (Aluko et al., 2009). At most of the pH values, oil droplet sizes for albumin-stabilized emulsions was significantly ($p \leq 0.05$)

higher (lower emulsion quality) than those of globulin-stabilized emulsions. The results indicate that the albumin is probably very hydrophilic with a smaller number of hydrophobic clusters as evidenced by the high solubility properties and sugar content, which probably limited interactions with the lipid phase and hence reduced emulsion forming ability. In contrast, the globulins have more hydrophobic clusters and interacted well with the lipid phase, which led to reduced droplet size (higher emulsion quality) of the emulsions when compared to albumin-stabilized emulsions. There was increased emulsion forming ability (smaller oil droplet sizes) of the albumins at pH 5 as compared to other pH values studied. Similar observation was reported previously for globulin and albumin fractions extracted from *Gingko biloba* seeds (Deng et al., 2011).

Emulsion stability is shown in Fig. 2.6, and the results suggest significant differences ($p<0.05$) for albumin and globulin at all pH values. For both fractions, emulsion stability increased from pH 3 to 5 indicating increased protein-protein interactions as a result of reduced net molecular charges, which enhanced formation of strong interfacial membranes. However, emulsion stability decreased at neutral pH suggesting weak interfacial membrane formation as protein-protein interactions are reduced due to increase in molecular net charge. Generally, the globulin-stabilized emulsions were very stable (>70%) at all pH values tested. This may be attributable to the high protein content (91.5%), and indicating the presence of relatively small oil droplets in globulin-stabilized emulsions when compared to albumin (40% protein). The relative instability observed in the albumin-stabilized emulsions is probably driven by unfavourable interactions (owing to the high hydrophilic carbohydrate component) between the oil droplets arising from the weak interfacial membranes. This type of

interfacial interactions tends to minimize the contact area between the two opposing phases by merging smaller droplets into larger ones. Eventually, the phases will separate, causing the emulsion to coalesce into larger ones, eventually leading to phase separation (Weiss, 2002).

2.4. Conclusion

The functional properties of the kidney beans albumin and globulin obtained in this study suggest its potential for the development of different food products. The albumin and globulin proteins in kidney bean proteins have shown high contents of lysine and arginine making this protein an important potential ingredient for food fortification. This is because dietary supplementation of foods with basic amino acids has been shown to normalize hormonal stress responses in human beings with high trait anxiety (Smriga, Ando, Akutsu, Furukawa, Miwa & Morinaga, 2007). The results obtained for the globulin, including the amino acid composition, protein solubility, WHC, foaming capacity, foam stability, emulsion capacity, and emulsion stability compared favorably with those reported for most legume seeds. As compared to the globulin, the albumin fraction did not exhibit as good foaming and emulsion stability, as well as gelation capacity. The ability of kidney bean globulin to form a gel at a lower concentration may be due to the higher surface hydrophobicity that facilitated increased protein-protein interactions better than the interactions between the albumin polypeptides.

Acknowledgments

Funding for this work was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a Discovery Grant to REA.

Table 2.1

Amino acid composition (%) of kidney bean flour, albumins and globulins

Amino acid	Flour	Albumin	Globulin
ASX	13.09	12.6	13.6
THR	4.52	6.43	3.57
SER	6.12	6.62	6.83
GLX	17.25	12.96	18.59
PRO	4.43	3.95	3.34
GLY	4.2	4.89	3.71
ALA	4.52	5.28	3.58
CYS	1.0	1.62	0.049
VAL	4.6	6.19	4.57
MET	1.36	1.3	0.62
ILE	3.9	4.22	4.47
LEU	8.13	7.53	9.3
TYR	2.94	2.95	3.87
PHE	5.89	5.49	7.26
HIS	2.97	2.51	3.16
LYS	7.5	9.05	7.29
ARG	6.32	4.5	5.62
TRP	1.17	1.89	0.53
e/t*	42.9	47.56	44.64

*e/t: ratio of essential to total amino acids

ASX: aspartic acid + asparagine GLX: glutamic acid + glutamine

Table 2.2

Physicochemical properties of kidney bean albumin and globulin proteins*

Parameter	Albumin	Globulin
Protein content (%)	41±0.00[b]	91.5±0.00[a]
Total carbohydrate content (%)	45±0.00[a]	3.9±0.00[b]
Least gelation concentration (%)	16±0.00 [a]	6.0±0.00 [b]
Water holding capacity (mL/g)	3.4±0.1 [a]	2.56±0.057[b]
Oil holding capacity (mL/g)	2.37±0.12 [a]	1.87±0.06 [b]
Total cysteine content (µmol/g)	1.12±0.5[a]	0.052±0.2[b]
Sulfhydryl [total (µmol/g)]	4.6± 0.029[a]	3.7± 0.001[b]
Sulfhydryl [exposed (µmol/g)]	2.2± 0.005[a]	1.4± 0.02[b]

*Results are mean ± SD of three replications; Different superscript letters within rows indicate that values showed statistically significant differences between samplese ($p < 0.05$).

Fig. 2.1. Protein solubility of kidney bean albumin and globulin fractions with changes at pH 2-9. Each data point represents average of triplicate measurements.

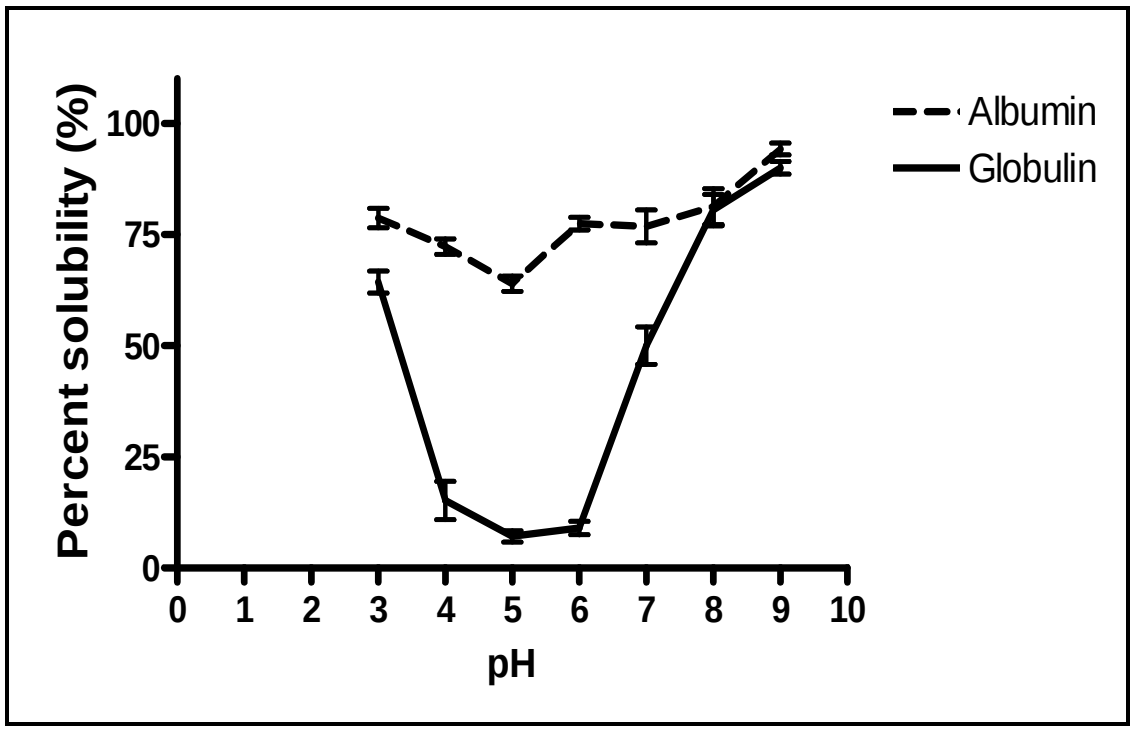


Fig. 2.2. SDS-PAGE analysis of kidney bean albumins and globulins showing the polypeptide composition of globulin (lane 2 and 4) and albumin (lane 3 and 5) under non-reducing (lane 4 and 5) and reducing (lane 2 and 3) conditions with lane 1 composed of standard proteins.

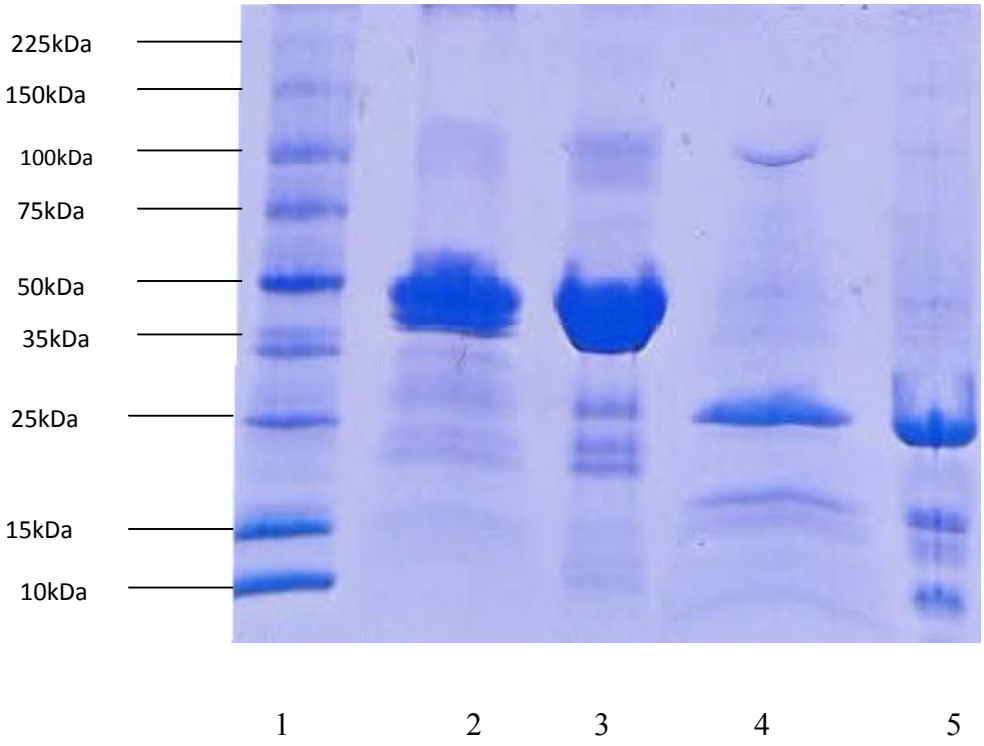
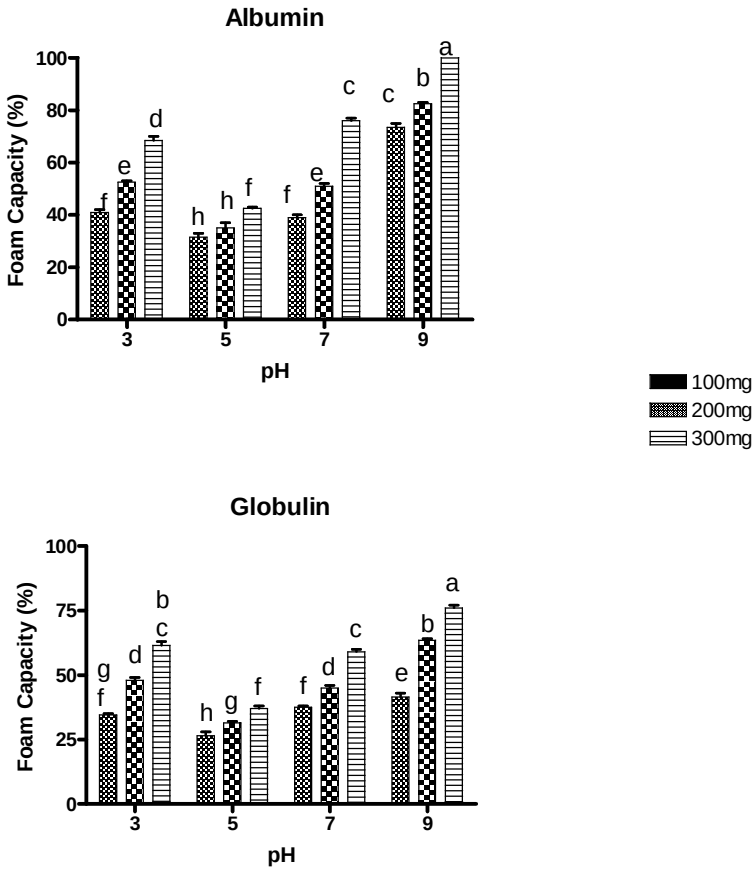
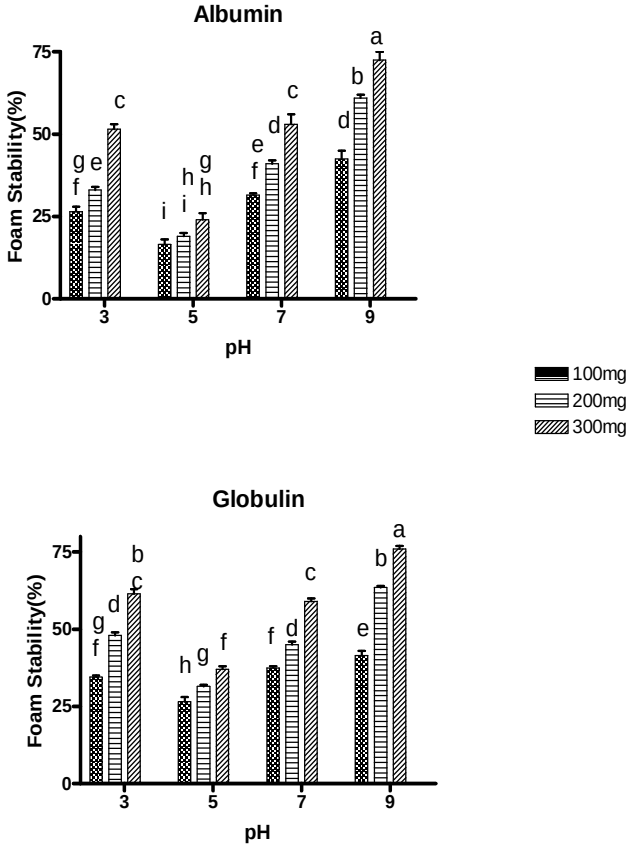


Fig. 2 3. Effect of pH and sample concentrations on the foaming capacity of kidney bean albumin and globulin fractions.



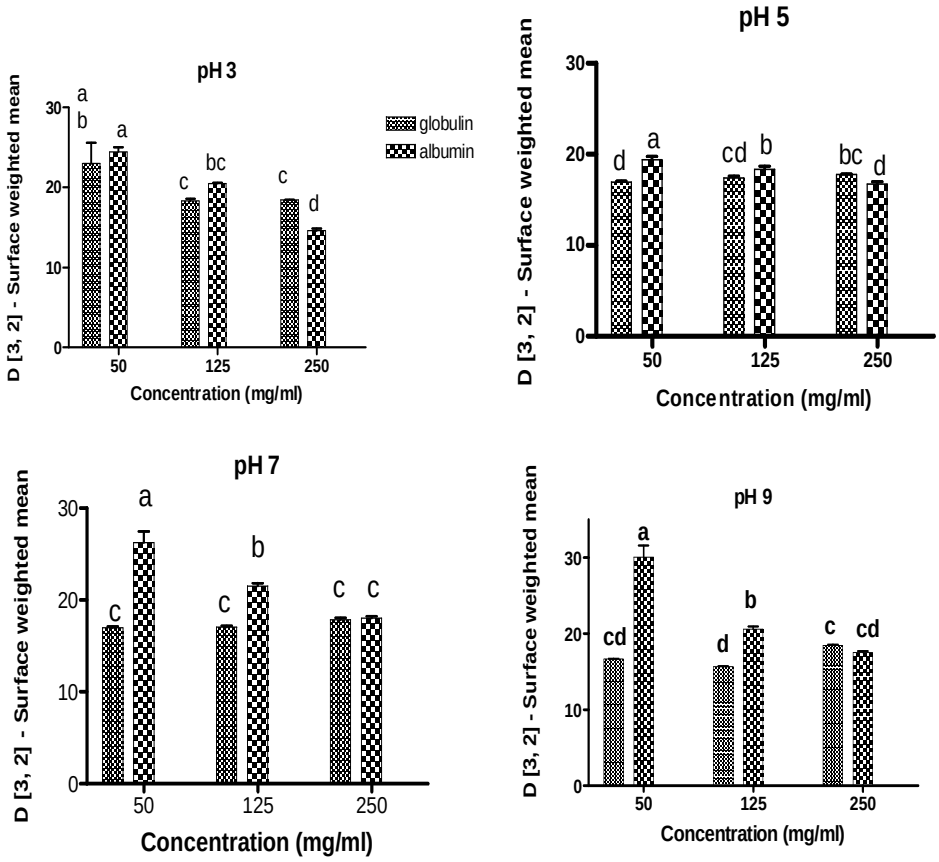
For each plot, bars with different letters are significantly different ($P \leq 0.05$)

Fig. 2.4. pH and concentration-dependent changes in foams stabilized by kidney bean albumin and globulin protein fractions. The foam stability is expressed as percentage of the original foam volume and measured after storage at room temperature for 30 min.



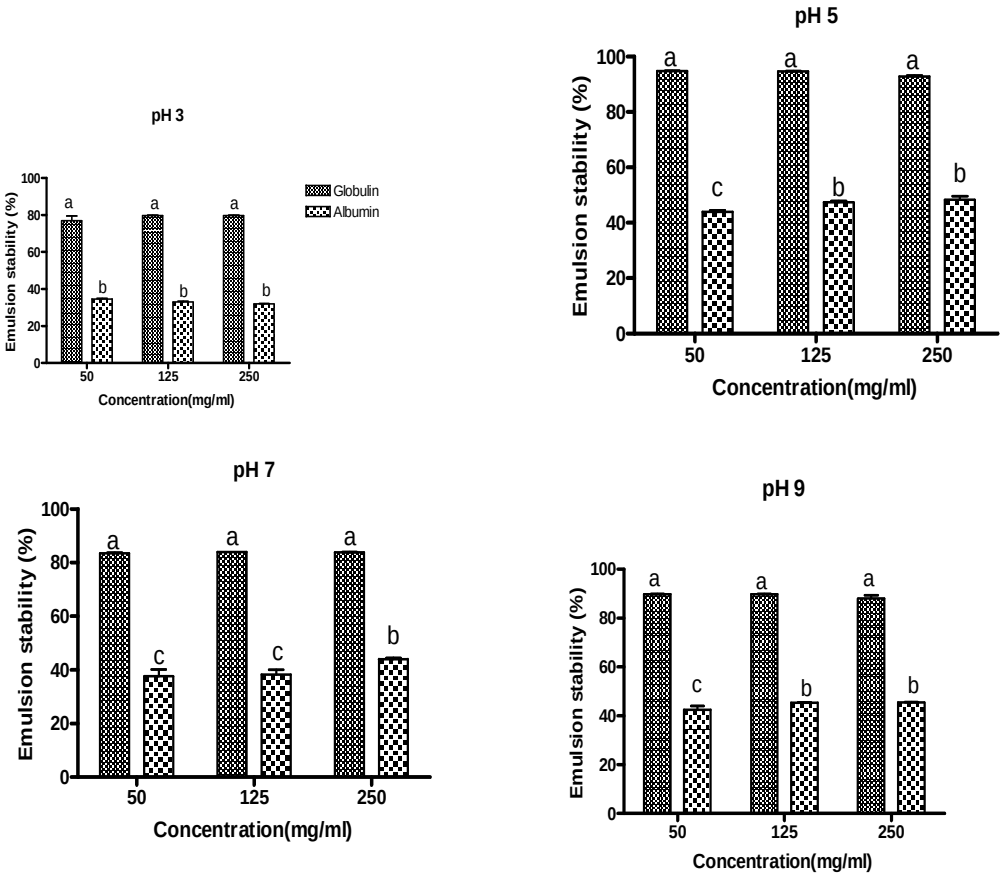
For each plot, bars with different letters are significantly different (P≤0.05)

Fig. 2.5. Effects of pH and protein concentration on the oil droplet size ($d_{3,2}$) of oil-in-water emulsions stabilized by kidney bean albumin and globulin protein fractions.



For each plot, bars with different letters are significantly different (P≤0.05)

Fig. 2. 6. Effects of pH and flour concentration on emulsion stability (increase in oil droplet size determined as the percentage ratio of initial to the 1 h $d_{3,2}$ values) made with kidney bean albumin and globulin proteins.



For each plot, bars with different letters are significantly different ($p < 0.05$).

References

Adebowale, Y. A., & Adebowale, K. O. (2007). Evaluation of the gelation characteristics of mucuna bean flour and protein isolate. *Electronic Journal of Environmental, Agricultural and Food Chemistry*, *6*, 2243-2262.

Eskin, M. & Tamir, S. (2006). Dictionary of nutraceuticals and functional food, Taylor & Francis, pp 234 and 243.

Aletor, O., Oshodi, A. A., & Ipinmoroti, K. O. (2002). Chemical composition of common leafy vegetables and functional properties of their leaf protein concentrate. *Food Chemistry, 78*, 63-68.

Aluko, R. E. (2004). The extraction and purification of proteins: an introduction. In: R. Y. Yada (Ed.), *Proteins in food processing* (pp. 323-346). Woodhead Publishing Limited and CRC Press LLC.

Aluko, R. E., & McIntosh, T. (2004). Electrophoretic and functional properties of mustard seed meals and protein concentrates. *Journal of the American Oil Chemists' Society*, *81*, 679-683.

Aluko, R. E., & Yada, R. Y. (1995). Structure-function relationships of cowpea *(Vigna unguiculata)* globulin isolate. Influence of pH and NaCl on physicochemical and functional properties. *Food Chemistry, 53*, 259-265.

Aluko, R. E. & McIntosh, T. (2001). Polypeptide profile and functional properties of canola meal. *Journal of the Science of Food and Agriculture*, *81*, 391-396**.**

Aluko, R. E., Mofolasayo, O. A., & Watts, B. M. (2009). Emulsifying and foaming properties of pea seed flours. *Journal of Agricultural and Food Chemistry, 57*, 9793-9800.

Beveridge, T., Toma, S. J., & Nakai, S. (1974). Determination of SH and SS-groups in some food proteins using Ellman's reagent. *Journal of Food Science, 39*, 49-51.

Beuchat, L. R. (1977). Functional and electrophoretic characteristics of succinylated peanut flour protein. *Journal of Agricultural and Food Chemistry*. *25*, 258–261.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984). Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography, 336*, 93-104.

Bora, P. S. (2002). Functional properties of native and succinylated lentil (*Lens culinaris Medik.*) globulins. *Food Chemistry, 77*, 171–176.

Brown, J. W. S., Ma, Y., Bliss, F. A., & Hall, T. C. (1981). Genetic variation in the subunit of globulin-1 storage protein of French bean. *Theoretical and Applied Genetics*, 59(2), 83–88.

Chagas, E.P. and Santoro, L.G. (1997). Globulin and albumin proteins in dehulled seeds of three *Phaseolus vulgaris* cultivars. *Plant Foods for Human Nutrition Dordrecht 51*, 17-26.

Chau, C. F., & Cheung, P. C. K. (1998). Functional properties of flours prepared from three Chinese indigenous seeds grown in China. *Food Chemistry*, 61, 429–433.

Hagas, E.P. And Santoro, L.G. (1997). Globulin and albumin proteins in dehulled seeds of three *Phaseolus vulgaris* cultivars. *Plant Foods for Human Nutrition, 51*, 17-26.

Chau, C. F., Cheung, K., & Wong, Y. S. (1997). Funcional properties of protein isolates from three Chinese indigenous legume seeds. *Journal of Agricultural and Food Chemistry, 45*, 2500-2503.

Chel-Guerrero, L., Pérez-Flores, V., Betancur-Ancona, D., & Dávila-Ortiz, G. 2002. Functional properties of flours and protein isolates from *Phaseolus lunatus* and *Canavalia ensiformis* seeds. *Journal of Agricultural and Food Chemistry, 50*, 584–591.

Deng, Q., Wang, L., Wei, F., Xie, B., Huang, F-H., Huang, W., Shi, J., Huang, Q., Tian, B., & Xue, S. (2011). Functional properties of protein isolates, globulin and albumin extracted from Ginkgo biloba seeds. *Food Chemistry*, *124*, 1458-1465.

Dietary Reference Intakes (DRI) for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (Macronutrients). (2005). Food and Nutrition Board.

Dzudie, T., & Hardy, J. (1996). Physicochemical and functional properties of flours prepared from common beans and green mung beans. *Journal of Agricultural and Food Chemistry, 44*, 3029-3032.

Foh, M. B. K., Amadou, I., Kamara, M. T., Foh, B. M., & Wenshui, X. W. (2011). Effect of enzymatic hydrolysis on the nutritional and functional properties of nile tilapia (*Oreochromis niloticus*) proteins. *American Journal of Biochemistry and Molecular Biology, 1*, 54-67.

Gehrke, C. W., Gehrke, L. L., Wall, J. S., Absheer, F. E., & Kaiser, Z. R. W. (1985). Amino acid analysis. *Journal of the Association of Official Analytical Chemists, 68*, 811-821.

Genovese, M. I., & Lajol, F. M. (1998). Influence of naturally acid-soluble proteins from beans (*Phaseolus vulgaris* L.) on in vitro digestibility determination. *Food Chemistry*, *62*, 315-323.

Grigelmo-Miguel, N., Gorinstein, S., & MartõÂn-Belloso, O. (1999). Characterisation of peach dietary fibre concentrates as a food ingredient. *Food Chemistry, 65*, 175-181.

Hang, Y. O., Steinkraus, K. H., & Hackler, L. R. (1980). Amino acid composition of high protein fractions prepared from mung beans, pea beans, and red kidney beans. *Journal of Food science*, *45*, 388-389.

Hou, Y., Hou, Y., Yanyan, L., Qin, G., & Li, J. (2010). Extraction and purification of a lectin from red kidney bean and preliminary immune function studies of the lectin and four Chinese herbal polysaccharides. *Journal of Biomedicine and Biotechnology,* 10.1155/2010/217342, http://dx.doi.org/10.1155/2010/217342.

Khalida, E. K., Babikerb, E. E., & EI-Tianay, A. H. (2003). Solubility and functional properties of sesame seed proteins as influenced by pH and/or salt concentration. *Food Chemistry*, 82, 361–366.

Kinsella, J. E. (1979). Functional properties of soy proteins. *Journal of the American Oil Chemists Society, 56*, 242-258.

Koening, R. L., Singh, S. P., & Gepts, P. (1990). Novel phaseolin types in wild and cultivated common bean (Phaseolus vulgaris, Fabaceae). *Economic Botany*, 44(1), 50–60.

Landry, J., & Delhaye, S. (1992). Simplified Procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. *Journal of Agricultural and Food Chemistry, 40*, 776-779.

Lawal, O. S., Adebowale, K. O., Ogunsanwo, B. M., Sosanwo, O. A., & Bankole, S. A. (2005). On the functional properties of globulin and albumin protein fractions and flours of African locust bean (*Parkia biglobossa*). *Food Chemistry, 92*, 681–691.

Lin, C. S., & Zayas, J. F. (1987). Functionality of defatted corn germ proteins in a model system: fat binding and water retention. *Journal of Food Science*, *52*, 1308–1311.

López, O. P., Falomir, C. O., & Vázquez, M. R. O. (1991). Chickpea protein isolates: physicochemical, functional and nutritional characterization. *Journal of Food Science, 56*, 3*,* 726-729.

Lqari, H., Vioque, J., Pedroche, J., & Millan, F. (2002). Lupinus angustifolius protein isolates: Chemical composition, functional properties and protein characterization. *Food Chemistry*, *76*, 349–356.

Markwell, M. A. C., Haas, S. M., Biebar, L. L., & Tolbert, N. E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and in protein samples. *Analytical Biochemistry*, *87*, 206-211.

Mahan, L. K., & Escott-Stump, S. (1996). Proteins. In: Krause's Food, Nutrition and Diet Therapy, W.B. Saunders Co., Philadelphia.

Meng, G., & Ma, C. Y. (2002). Characterization of red bean globulin. *International Journal of Food Science and Technology*, *37*, 687–695.

Montoya, C. A., Gomez, A.S., Lalle`s, J-P.,Souffrant, W.B. & Leterme, S.B.P. (2008). In vitro and in vivo protein hydrolysis of beans (*Phaseolus vulgaris*) genetically modified to express different *phaseolin* types. *Food Chemistry* 106 1225–1233.

Njintang, N, Y., Mbofung, C. M. F., & Waldron, K. W. (2001). In vitro protein digestibility and physicochemical properties of dry red bean (*Phaseolus vulgaris*)

flour: Effect of processing and incorporation of soybean and cowpea flour. *Journal of Agricultural and Food Chemistry*, *49*, 2465–2471.

Okezie, B. O., & Bello, A. B. (1988). Physicochemical and functional properties of winged bean flour and isolate compared with soy isolate. *Journal of Food Science*, *53*, 450-454.

Pusztai, A. And Palmer, R. (1977). Nutritional evaluation of kidney beans (Phaseolus vulgaris): The toxic principle. *Journal of the Science of Food and Agriculture, 28 (7),* 620-623.

Ragab, D. D. M., Babiker, E. E., & Eltinay, A. H. (2004). Fractionation, solubility and functional properties of cowpea (*Vigna unguiculata*) proteins as affected by pH and/or salt concentration. *Food Chemistry, 84*, 207–212.

Sai-Ut, S., Ketnawa, P., & Rawdkuen, S. (2009). Biochemical and functional properties of proteins from red kidney, navy and adzuki beans. *Asian Journal of Food and Agro-Industry, 2*, 493-504.

Salmanowicz, B. P. (2001). Phaseolin seed variability in common bean (Phaseolus vulgaris) by capillary gel electrophoresis. Journal of Applied Genetics, 42, 269–281.

Sanchez-Vioque, R., Vioque, C. J., Bautista, J., & Millan, F. (1999). Protein isolates from chickpea (*Cicer arietinum* L.): chemical composition, functional properties and protein characterization. *Food Chemistry, 64*, 237–243.

Sathe, S. K. (2002). Dry bean protein functionality. *Clinical Reviews in Biotechnology*, *22*, 175-223.

Schmidt, R. H. (1980). Gelation and coagulation. *ACS Symposium Series, 147,* 131–145.

Shi J., Xue S. J., Kakuda Y., Ilic S., & Kim, D. (2007). Isolation and characterization of lectins from kidney beans (*Phaseolus vulgaris*). *Process Biochemistry*, *42*, 1436-1442.

Smriga, M., Ando, T., Akutsu M., Furukawa, Y., Miwa, K., & Mo-rinaga, Y. (2007). Oral treatment with L-lysine and L-arginine reduces anxiety and basal cortisol levels in healthy humans. *Biomedical Research*, *28*, 85-90.

Tang, C. H. (2008). Thermal denaturation and gelation of vicilin-rich protein isolates from three Phaseolus legumes: A comparative study. LWT-*Food Science and Technology*, *41*, 1380–1388.

Tang, C. H., Sun, X., Yin, S. W., & Ma, C. Y. (2008). Transglutaminase-induced cross-linking of vicilin-rich kidney protein isolates: Influence on the functional properties and in vitro digestibility. *Food Research International, 41*, 941–947.

Tang, C. H. & Ma, C. Y. (2009). Heat-induced modifications in the functional and structural properties of vicilin-rich protein isolate from kidney (*Phaseolus vulgaris L.)* bean. *Food Chemistry*, *115*, 859–866.

Vasconcelos, I. M., Maia, F. M. M., Farias, D. F., Campello, C. C., Carvalho, A. F. U., Moreira, R. A., & Oliveira, J. T. A. (2010). Protein fractions, amino acid composition and antinutritional constituents of high-yielding cowpea cultivars. *Journal of Food Composition and Analysis, 23*, 54–60.

Weiss, J. (2002). Emulsion Stability Determination. In: R.E. Wrolstad. *Handboook of food analytical chemistry,* John Wiley & Sons, Inc. pp. 591-607.

Wu, W., Williams, W. P., Kunkel, M. E., Acton, J. C., Huang, Y., Wardlaw, F. B., & Grimes, L. W. (1996). Amino acid availability and availability-corrected amino

acid score of red kidney beans (*Phaseolus vulgaris L.)*. *Journal of Agricultural and Food Chemistry*, *44*, 1296–1301.

Yem, E. W., & Willis, A. J. (1954). The estimation of carbohydrates in plant extracts by anthrone. *Biochemistry Journal, 57*, 769-773.

Yin, S. W., Tang, C. H., Wen, Q. B., Yang, X. Q., & Li, L. (2008). Functional properties and *in vitro* trypsin digestibility of red kidney bean (*Phaseolus vulgaris L*.) protein isolate: Effect of high-pressure treatment. *Food Chemistry*, *110*, 938-945.

Yin, S-W., Tang, C-H., Wen, Q. B., & Yang, X-Q. (2010). Functional and conformational properties of phaseolin (*Phaseolus vulgris* L.) and kidney bean protein isolate: A comparative study. *Journal of the Science of Food and Agriculture, 90(4)*, 599–607.

Zhang, J., Shi, J., Ilic, S., Xue, S.J. & Kakuda, Y. (2008). Biological properties and characterization of lectin from red kidney bean (*Phaseolus vulgaris*). *Food Reviews International,* 25, (1), 1-16.

CHAPTER 3

EFFECTS OF NaCl AND pH VARIATIONS ON THE STRUCTURAL CONFORMATIONS OF KIDNEY BEAN VICILIN

Sule Mundi[a] and Rotimi E. Aluko[a,b*]

[ab]Department of Human Nutritional Sciences and the Richardson Centre for Functional Foods and Nutraceuticals, 196 Innovation Dr., Winnipeg, MB R3T 2N2.Canada

Corresponding Author, Tel.: 1204 4749555, Fax: 1(204) 474-7552

Email: alukor@cc.umanitoba.ca (Aluko, R.E)

Submitted to International Journal of Molecular Sciences

3.0. Abstract

Structural changes as a result of variations in pH value and salt concentration were determined for purified vicilin, the major globular protein in kidney beans using intrinsic fluorescence and circular dichroism (CD). The vicilin consisted of two polypeptide chains of about 43 and 45 kDa in size when analyzed under reducing SDS-PAGE. Intrinsic fluorescence measurements were performed to measure exposure of phenylalanine, tyrosine and tryptophan as a means of estimating protein conformational changes. Generally, the vicilin structure was highly denatured as shown by the extensive red shift (>350 nm) of the wavelength of maximum tryptophan fluorescence intensity. At pH 3.0 and 5.0, the fluorescence intensity (FI) was greater than values obtained at pH 7.0 and 9.0, which suggests that the microenvironment of the aromatic amino acids was less hydrophilic at acidic pH. Addition of NaCl also led to increased FI, an indication of structural changes that reduced the interaction of aromatic acids with hydrophilic compounds. These changes in FI were due mostly to tryptophan emission because tyrosine emission was suppressed. The far-UV CD spectra showed that vicilin had minimal measurable secondary structures at pH 3.0 and 5.0 when compared to pH 7.0 and 9.0. Addition of NaCl led to improvements in the tertiary structure of vicilin as determined from the near-UV CD spectra.

Keywords: Kidney bean; vicilin; fluorescence intensity; tryptophan; circular dichroism; amino acid composition

3.1. Introduction

Pulses, particularly kidney beans (*Phaseolus vulgaris L.*), are high in dietary fibre and low in fat, making beans a good source of cholesterol and blood sugar lowering food, which can reduce the risk of cardiovascular diseases and type 2 diabetes (Aykroyd & Doughty, 1982). In addition, they are high in proteins (20–30% on a dry weight basis) (Yin, Tang, Wen, & Yang, 2010a), which puts them among some of the richest food sources of proteins for human and animal nutrition, as well as for food industry applications. The main storage protein in kidney beans is the globular phaseolin, also known as vicilin (Yin et al., 2010a). Phaseolin is a 7S glycoprotein that reversibly and non-enzymatically binds to specific sugars, and in doing so, plays a range of crucial roles in many cell–cell recognition events that trigger several important cellular processes (Chandra et al., 2006). Glycoproteins are able to recognize diverse sugar structures and have the ability to mediate a variety of biological processes such as cell–cell and host–pathogen interactions, serum–glycoprotein turnover, as well as innate immune responses (Vijayan & Chandra, 1999). As a consequence of specific cell recognition ability, several biological effects such as antitumor, immunomodulatory, antifungal, and anti human immunodeficiency virus (HIV) have been associated with kidney bean proteins (Eskin & Tamir, 2006). All of the biological functions reported for proteins are directly affected by the structure and other physical properties such as size, charge and hydrophobicity of the protein, which affects interactions with or affinity for ligands (Chandra et al., 2006). Changes in the protein conformation and biological functions can occur as a result of alterations in composition of the surrounding environment. For example, the presence of protein structure perturbants (e.g. sodium dodecyl sulfate, urea and dithiothreitol) and

other environmental parameters, such as pH, ionic strength, binding of ligands and temperature can affect molecular organization, which influences biological functions. Thus, understanding the impact of protein conformation under various buffer conditions on the biological functions remains a primary focus in protein chemistry, especially during various food processing events. Cooking and other forms of food preparation or processing are usually necessary for the reduction of anti-nutrient factors in kidney beans (Shimelis & Rakshit, 2007). During processing events, the conditions in the food environment changes and hence, the structure could be affected. The knowledge of changes in temperature, pH, or ionic strength, and their effects on structure-function relationships as well as their control during food preparation is essential. Spectroscopic methods, including circular dichroism (CD) and fluorescence spectroscopy (FS) of proteins can provide sensitive indications of the conformational changes that may occur (at the molecular level) due to changes in the protein environment. CD and FS of proteins provide important structural information concerning the details of the helical content of proteins or the asymmetric environment of aromatic residues. Previous studies (Dyer, Nelson, & Murai, 1992; Tang & Ma, 2009; Tang, Sun, & Foegeding, 2011; Yin et al., 2010a; Yin, Tang, Wen, Yang, & Yuan, 2010b; Yin, Huang, Tang, Yang, Wen, & Qi, 2011a; Yin, Tang, Yang, & Wen, 2011b) have investigated the effects of protein structure perturbants and pH on the conformational properties of phaseolin and kidney bean isolate (KPI). By using CD, FS and differential scanning calorimetry (DSC), conformational and structural changes due to heat (Dyer et al., 1992; Tang & Ma, 2009), succinylation and acetylation (Yin et al., 2010b), glycation (Tang et al., 2011), polar, neutral, anionic and cationic quenchers such as acrylamide, nitrate and caesium ion respectively (Yin et al.,

2011b) have been studied. The results showed significant changes in the α-helix content and highly ordered secondary structures (α-helix + β-strand) in addition to tertiary conformation unfolding and subsequent rearrangement process. Yin et al. (2011a) studied the effects of pH on the secondary and tertiary conformation of phaseolin by using CD spectra in near ⁄ far UV region with the result shown changes in conformation with shift in pH. However, our understanding of the combined effects of varying ionic strength and pH on the CD and FS properties of purified kidney bean globular protein (vicilin) is still limited.

The goal of the current research therefore, was to study the effects of changes in pH alone or in combination with NaCl concentrations on the structural conformations of purified kidney bean vicilin using CD and FS.

3.2. Materials and Methods

3.2.1 Preparation of crude globulin sample

Red kidney bean seeds were obtained from a local store in Winnipeg and were ground into flour using a Retsch ZM200 centrifuge mill (Retsch GmbH, Haan, Germany). Globulin proteins were extracted from the flour according to the previously described ammonium sulfate precipitation method (Aluko, 2004). The major globulin fraction in kidney beans was obtained by adjusting an aqueous extract (obtained using 0.1 M phosphate buffer, pH 7.0 containing 0.4 M NaCl) of the flour to 40% ammonium sulfate saturation.. After centrifugation (9000 x *g*, 45 min, 4^{o}C), the supernatant was then brought to 80% ammonium sulfate saturation to precipitate the globulins. The ammonium sulfate was then removed from the isolated globulins by dialyzing sample against water at 2^{o}C using a membrane with 6-8 kDa molecular weight cut-off. The dialysis bag

content was centrifuged (9000 x *g*, 45 min, 4^{o}C) and the resultant precipitate (globulin) was freeze-dried and further purified using Fast Protein Liquid Chromatography (FPLC).

3.2.2. Purification of vicilin by fast protein liquid chromatography (FPLC)

The kidney beans globulin protein was separated using ÄKTAPurifier FPLC system equipped with a size exclusion column, HiLoad 26/60 Superdex 200 Prep grade (GE Healthcare, Montreal, PQ). Sodium phosphate (0.1 M) prepared to contain 0.4 M NaCl was used as the equilibration buffer for the column, and as an elution buffer. Two millilitres of 100 mg/mL globulin protein solution, filtered through 0.2 μm filter disks was loaded onto the Superdex column and run at a flow rate of 2.5 mL/min; eluted proteins were detected from the UV absorbance at 214 nm. The major fraction was collected and desalted using Hiprep 26/10 desalting column, following which the sample was freeze dried, analyzed for protein content (Markwell, Haas, Bieber, & Tolbert, 1978) and stored at −20 °C until further analysis.

3.2.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The freeze dried globulin fraction was subjected to SDS-PAGE according to the method of Aluko & McIntosh (2004) with minor modifications. Ten milligram protein was dispersed in a Tris/HCl buffer containing 10% (w/v) SDS, followed by heating at 95^{o}C for 10 min, cooling and centrifugation (15000 x *g*) for 5 min. After centrifugation, approximately 1 μL of supernatant was loaded onto an 8-25% gradient gel and electrophoresis performed with a Phastsystem Separation and Development unit according to the manufacturer's instructions (GE Healthcare, Montréal, PQ).

3.2.4. Amino acid analysis

HPLC system was used for the analysis of the amino acid profiles after samples were hydrolyzed with 6 M HCl according to the method described by Bidlingmeyer et al. (1984) (Bidlingmeyer, Cohen, & Tarvin, 1984) The cysteine and methionine contents were determined after performic acid oxidation (Gehrke, Wall, & Absheer, 1985) and tryptophan content was determined after alkaline hydrolysis (Landry & Delhaye, 1992).

3.2.5. Intrinsic fluorescence emission spectrophotometry

Intrinsic fluorescence spectra were recorded on the JASCO FP-6300 spectrofluorimeter at 25°C using a 1 cm pathlength cuvette. Protein stock solution was diluted to 0.002% (w/v) and fluorescence spectra recorded at excitation wavelengths of 275 (tyrosine and tryptophan) and 295 (tryptophan) with emission recorded from 280 and 300 to 500 nm, respectively. Emissions of the buffer blanks were subtracted from those of the respective samples to obtain fluorescence spectra of the sample.

3.2.6. Measurements of circular dichroism (CD) spectra

CD measurements were carried out at 25^{o}C in a J-810 spectropolarimeter (JASCO, Tokyo, Japan) using the spectral range of 190-240 nm (far UV) for secondary structure determinations and 250-320 nm (near UV) for tertiary structure. The vicillin was solubilized in 10 mM phosphate buffer that contained appropriate NaCl concentration followed by centrifugation at 10000xg for 30 min and supernatant used for CD structural analysis. For secondary structure determinations, a cuvette with pathlength of 0.05 cm was used and contained 1 mg/mL protein solution while the tertiary structure was measured in a 0.1 cm cuvette that contained 2 mg/mL protein concentration. All the

CD spectra were obtained as the average of three consecutive scans with automatic subtraction of the buffer spectra.

3.3. Results and Discussion

3.3.1. FPLC purification and gel electrophoresis

Kidney beans globulin proteins were separated based on the differential exclusion from the pores of the packing material of the column. Figure 3.1A shows that the FPLC fractionation resulted in one major peak, the 7S vicilin and a minor fraction, probably a phytohemagglutinin (Sathe, 2002). The major fraction was collected, freeze-dried and used for the structural characterization reported in this study. SDS-PAGE was used to separate proteins according to their electrophoretic mobility as a function of polypeptide molecular weight (MW). The gel electrophoresis under reducing condition (Figure 3.1B) revealed bands with electrophoretic patterns that supported the FPLC profile of the fractions obtained. A major polypeptide band with estimated MW of 45 kDa was present in the purified (lane 2) and crude (lane 3) proteins. A minor polypeptide band corresponding to 27-28 kDa was also observed for the crude globulin preparation (lane 3), which is probably the minor protein peak observed in the FPLC chromatogram (Figure 3.1A). Since the works of Osborne (1894), it has been known that the extractable proteins in beans are mostly globulins with two or more (Joubert, 1957; Waterman, Johns, & Jones, 1923) polypeptides reported for kidney bean proteins. Electrophoretic patterns similar to the major band reported in this study with MW of 45 kDa have also been reported for red kidney beans 7S vicilin (Bollini & Chrispeels, 1978; Hou, Hou, Yanyan, Qin, & Li, 2010). The 7S vicilin are also known by several other names by earlier authors as phaseolin (Osborne, 1894; Waterman et al., 1923) fraction E (Jaffé &

Hannig, 1965; Pusztai & Watt, 1970), glycoprotein II (Pusztai & Watt, 1970), G1 fraction (Sun & Hall, 1975), α component (Ishino & Ortega, 1975),7S component (Vaintraub, Bassüner, & Shutov, 1976). In addition, other globulins such as the phytohemagglutinins (lectins) with molecular weights of 28-30 kDa (Shi, Xue, Kakuda, Ilic, & Kim, 2007) and fractions with toxic properties (Jaffé & Hannig, 1965) have been purified from beans.

3.3.2. Amino acid analysis

As shown in Table 3.1, the kidney bean protein revealed high level of aspartic acid+asparagine and glutamic acid+glutamine, indicating the high content of amides which serve a nitrogen storage role in the seeds. Glutamine is also important in human nutrition because it promotes glycogen production in the liver, increases protein synthesis and decreases protein degradation in skeletal muscle (Franzese, 2000). The high contents of lysine and arginine in the kidney bean proteins may provide important source of ingredients for food fortification. This is because dietary supplementation of foods with basic amino acids has been shown to normalize hormonal stress responses in human beings with high anxiety trait (Smriga et al., 2007). As in other legumes, the nutritionally limiting amino acids found in the kidney bean proteins are the sulfur-containing amino acids (methionine and cysteine). The content of branched-chain amino acids (leucine + isoleucine + valine) was slightly increased (from 17.33 to 18.34%) after FPLC purification. Proteins with high levels of branched-chain amino acids are appropriate for formulating physiologically active foods, including diets that are designed to improve nutritional status of patients with medical conditions such as burns, cancer, liver failure, and trauma (Weisdorf, 1998). The percentage ratio of the essential to total amino acids was above the 35% value that is reported for ideal proteins (Panel on Dietary Reference

Intakes for Electrolytes and Water & National Academies Press, USA, 2005). The purified kidney bean vicilin showed a low lysine/arginine (L/A) ratio (1.44), which is comparable to that of fish proteins but is lower than those of casein (L/A = 1.89) and whole milk protein (L/A = 2.44). A low lysine/arginine ratio is believed to have less lipidemic and atherogenic effects (Wanasundara & Shahidi, 2003).Thus the kidney bean 7S globulin may be a better choice for formulating hypolipidemic diets when compared to casein (L/A = 1.89) and whole milk (L/A = 2.44).

3.3.3. Intrinsic Fluorescence

The emission maximum of the fluorescent amino acids such as tyrosine and tryptophan in their specific environment could be a useful signature because it provides a sensitive means of characterizing proteins based on their conformation, dynamics, and intermolecular interactions (Pain, 1996). Protein fluorescence can reveal a variety of information, including the extent of rotational freedom, the exposure of amino acid side chains to quenchers, and intramolecular distances (Gorinstein et al., 2000). Therefore, changes in intrinsic fluorescence can be used to monitor structural conformations of proteins. In the current work, we measured protein fluorescence as a function of pH and NaCl concentrations. The monitoring of the changes in pH and NaCl is important as they may affect solubility and the polarity of the local environment. As a possible food ingredient, vicilin-rich kidney bean globulin will be subjected to pH and salt changes during food manufacturing, leading to conformational and structural changes which could eventually impact the nutritional and functional properties (Yin et al., 2011b).

The fluorescence chromatograms shown in Fig. 3.2 and 3.3 relate to fluorescence intensity (FI) obtained after excitations at 295 (tryptophan) and 275 (tryptophan +

tyrosine) nm, respectively. Changes in pH can affect the overall charge on the ionizable proteins such as arginine, histidine, lysine, glutamic acid and aspartic acid, and consequently alter electrostatic interactions which may change protein conformation (Yin et al., 2011b). Figures 3.2 and 3.3 show quenching of the fluorescence intensity at neutral and alkaline pH values, which can be attributed to protein unfolding that leads to increased interactions with the hydrophilic environment (Arntfield, Ismond, & Murray, 1987). Thus the vicilin protein had a more compact structure at pH 3.0 and 5.0, which reduced intramolecular quenching of aromatic amino acid fluorescence (Stanciuc, Rapeanu, Bahrim, & Aprodu, 2012). Addition of NaCl generally led to increased FI, which indicates salt-induced changes in protein structure to reduce hydration of aromatic amino acids and associated quenching of aromatic amino acid fluorescence. Table 3.2 shows data from excitation at 295 nm, which can be used to estimate the degree of polarity of tryptophan microenvironment within the vicilin molecule. The wavelength of maximum FI (λmax) was >350 nm for all the samples, which indicates that the tryptophan residues were positioned within a highly hydrophilic environment. This is because in a hydrophobic environment, tryptophan λmax is typically between 331-347 nm (Arntfield et al., 1987). The present results are different from the λmax value of 331 nm reported by Yin et al. (2011a & b), probably due to differences in extraction and purification methods. In the absence of NaCl, the tryptophan microenvironment became less hydrophilic as evidenced by the blue shift in wavelength of maximum fluorescence (λmax) from 368 nm at pH 3.0 to 360 at pH 9.0. The data suggest that the tryptophan residues became gradually buried within the inside of the protein as the environment became more hydrophilic with increase in pH values. Addition of NaCl at pH 3.0 led to

gradual transfer of the tryptophan residues away from the hydrophilic environment as shown by the blue shift from 368 nm for 0.0 NaCl to 362 and 364 nm for 0.5 and 1.0 NaCl, respectively (Table 3.2). The blue shift in λmax was accompanied by increased FI as NaCl increased at pH 3.0. The results suggest that at pH 3.0, the presence of NaCl could have attracted water molecules away from the protein core, which reduced quenching of tryptophan fluorescence. In contrast, there were no changes in λmax at pH 7.0 while only the addition 0.5 M NaCl led to a red shift at pH 5.0 and 9.0 (Table 3.2). Overall, the highest tryptophan fluorescence was observed at pH 5.0, which is close to the isoelectric point of the vicilin where a more compact structure could have reduced both intramolecular distance between the tryptophan molecules and fluorescence quenching by the hydrophilic molecules. But as pH was increased to 7.0 and 9.0, the overall tertiary structure of the vicilin became less compact, which led to increased rotational freedom (usually associated with longer intramolecular distances between tryptophan residues) and hence reduced FI intensity (Stanciuc et al., 2012). The tryptophan λmax for fully denatured proteins has been reported to be ~354 nm (Stanciuc et al., 2012); therefore, the values obtained in this work suggest a highly denatured state for the proteins within a strong hydrophilic environment. Table 3.3 shows similar data such as the blue shift in λmax and reduced FI as the pH was increased. The typical λmax for tyrosine is ~303 nm when excited at 275 nm; thus the longer λmax values reflect tryptophan fluorescence and not tyrosine (Table 3.3). Due to the stronger fluorescence quantum yield of tryptophan and its ability to receive transfer of non-radiative energy, it is recognized that tyrosine fluorescence (303 nm) is barely detectable in the presence of tryptophan (Schmid, 1989). The present data is different from those reported by Yin et al.

(2011a) showing detection of maximum tyrosine fluorescence at 318 nm, possibly as result of differences in protein preparation methods.

3.3.4. Circular dichroism

Signals arising from chromophores, including the amide group of the polypeptide backbone with associated peaks falling in the far UV region (190-240nm), and the aromatic residues (Phe, Tyr, and Trp) around the near UV region (250-320 nm) of the spectrum, makes CD suitable for examining protein conformation and folding under a wide range of experimental conditions. In the current study, conformational changes of kidney bean 7S vicilin were investigated as a function of changes in pH and NaCl concentrations. Figure 3.4 shows that changes in the tertiary structure of vicilin were influenced more by NaCl content than by pH. In the absence of NaCl (0.0 M), the vicilin had minor tertiary structures at different pH values as evident in the CD values being very close to zero. Addition of NaCl led to significant ($p<0.05$) changes in the tertiary structure mostly at pH 3.0 in the presence of 0.1 and 1.0 M concentrations. However, the secondary structure data showed loss of structures especially at acidic pH values in contrast to pH 7.0 and 9.0 where conformations were more evident (Fig.3.5). At pH 5.0, no secondary structure transitions were observed in the presence or absence of NaCl, which suggests a highly denatured protein at the isoelectric point (Sathe, 2002). Addition of 0.1 M NaCl led to significant secondary structure development at pH 3.0; increases in NaCl to 0.5 and 1.0 M had opposite effects in eliminating the secondary structure conformations (Fig. 3.5). The secondary structure conformations showed the presence of the typical β-sheet (peak at 195 nm) and α-helix (peak at 215 nm) transitions for the vicilin in the absence of NaCl (0.0 M) at pH 7.0 and 9.0. Tang et al. (2011) and Yin et al.

(2011a) also observed prominent negative band at pH 7.0, around 215-220 nm and a positive band at about 195-200 nm in the far-UV spectra of vicilin purified using DEAE-Sepharose fast flow column chromatography. In our work, addition of NaCl reduced these β-sheet and α-helix transitions, which suggests salt-induced structural denaturation of the vicilin. It is possible that the presence of NaCl introduced excess ionic charges which increased protein-protein repulsion at the secondary structure level.

3.4. Conclusions

Intrinsic fluorescence studies of red kidney bean vicilin showed extensive denaturation of protein structure as evident in the tryptophan fluorescence with λmax values >350 nm. Tryptophan fluorescence quenching was higher at neutral and basic pH values, which indicates higher polarity of the microenvironment when compared to acidic pH values. The structural conformations of the vicilin suggest close proximity of tyrosine to tryptophan because of the absence of tyrosine emission at all the pH values and NaCl concentrations. In the presence of NaCl, tryptophan fluorescence was enhanced, which suggests salt-induced reduction in the intramolecular distance between the aromatic amino acid residues. Circular dichroism studies showed complete lack of defined secondary structure of the vicilin at pH 3.0 and 5.0, which is close to the isoelectric point. At pH 7.0 and 9.0, the vicilin assumed a more defined secondary structure conformation that was sensitive to changes in NaCl concentrations. The vicilin showed minimal tertiary structure conformations at all the pH values tested but more defined structures were produced upon addition of NaCl. Overall, the purification methods led to substantial denaturation of vicilin protein that was partially ameliorated in the presence of NaCl.

Acknowledgements

Funding for this work was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a Discovery Grant to REA.

Table 3.1: Amino acid composition of crude and purified red kidney bean vicilin*

Amino acid	Purified vicilin	Crude vicilin
ASP	13.58	13.6
THR	3.03	3.57
SER	6.21	6.83
GLU	21.34	18.59
PRO	3.40	3.34
GLY	3.34	3.71
ALA	3.51	3.58
CYS	0.01	0.05
VAL	4.03	4.57
MET	0.93	0.62
ILE	4.07	4.47
LEU	9.23	9.30
TYR	3.84	3.87
PHE	7.18	7.26
HIS	2.89	3.16
LYS	7.39	7.29
ARG	5.11	5.62
TRP	0.90	0.53
BCAA	17.33	18.34
AAA	11.92	11.66
BCCA/AAA	1.50	1.60
EAA	44.62	45.98

*BCAA- branched chain amino acid, AAA-aromatic amino acids, EAA- essential amino acid

Table 3.2

Effects of pH and NaCl on intrinsic fluorescence intensity (FI) and wavelength of maximum FI (λmax) of red kidney bean vicilin excited at 295 nm (tryptophan)

NaCl	pH 3.0		pH 5.0		pH 7.0		pH 9.0	
(M)	λmax (nm)	FI	λmax (nm)	FI	λmax (nm)	FI	λmax (nm)	FI
0	368	108.1	368	124.5	362	97.7	360	76.1
0.1	366	153.5	368	200.	362	134.8	360	105.0
0.5	362	204.9	370	289.6	362	111.2	364	124.4
1.0	364	201.8	368	266.3	362	97.3	360	115.7

Table 3.3

Effects of pH and NaCl on intrinsic fluorescence intensity (FI) and wavelength of maximum FI (λmax) of red kidney bean vicilin excited at 275 nm (tyrosine+tryptophan)

NaCl	pH 3.0		pH 5.0		pH 7.0		pH 9.0	
(M)	λmax (nm)	FI	λmax (nm)	FI	λmax (nm)	FI	λmax (nm)	FI
0	362	72.9	360	69.4	358	65.2	356	56.5
0.1	358	289.2	360	166.5	358	122.7	358	98.4
0.5	358	167.6	360	205.7	358	90.2	358	84.7
1.0	362	133.9	360	137.2	358	97.9	356	91.0

Fig. 3.1 (A) Fast protein liquid chromatography (FPLC) profile of kidney bean globular protein purified using HiLoad 26/60 Superdex 200 prep grade column and 0.1 M sodium phosphate (pH 8) as elution buffer. (B) Reducing SDS-PAGE profiles of red kidney bean globular protein. Lane 2 and 3 are the bands for purified and unpurified globulin, respectively. Lane 1 is the promega standard proteins.

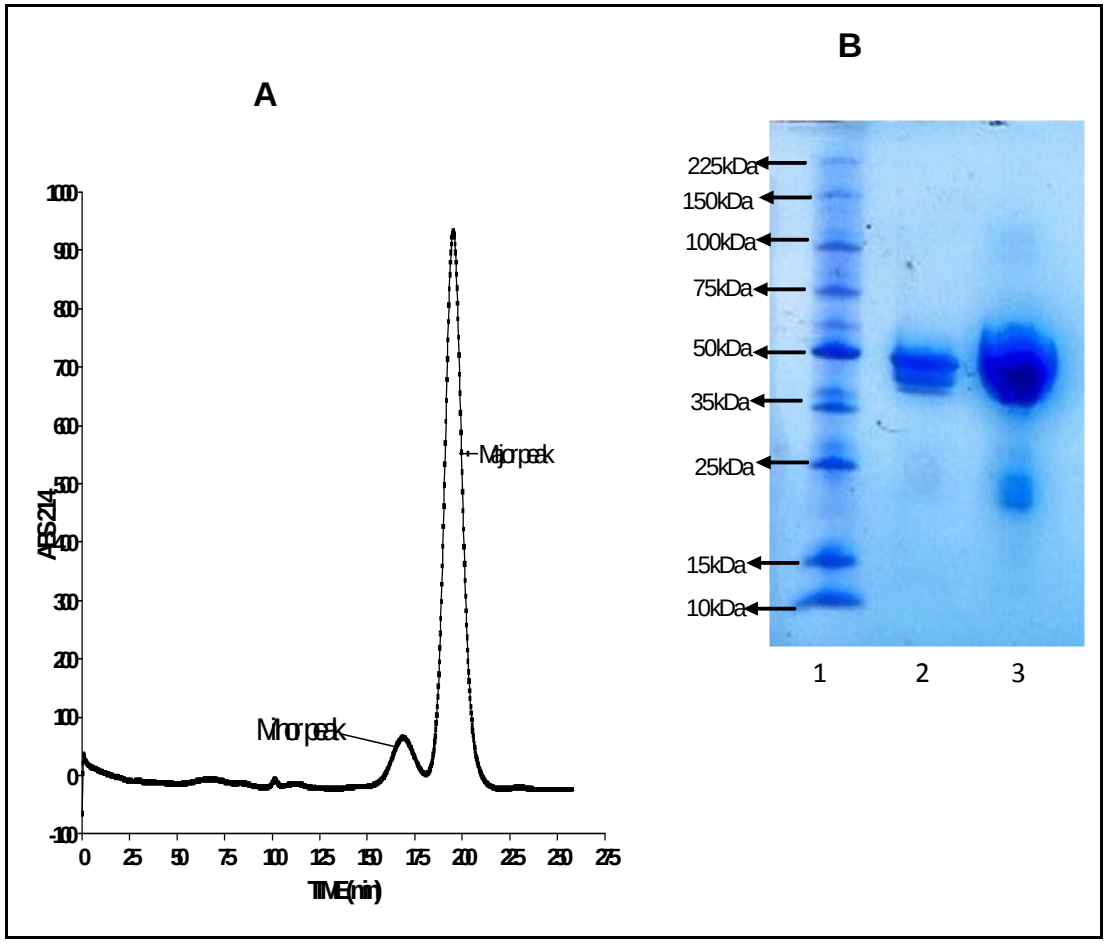


Fig. 3.2 The intrinsic fluorescence spectra of kidney bean vicilin excited at 295 nm (tryptophan) as affected by pH and salt concentration.

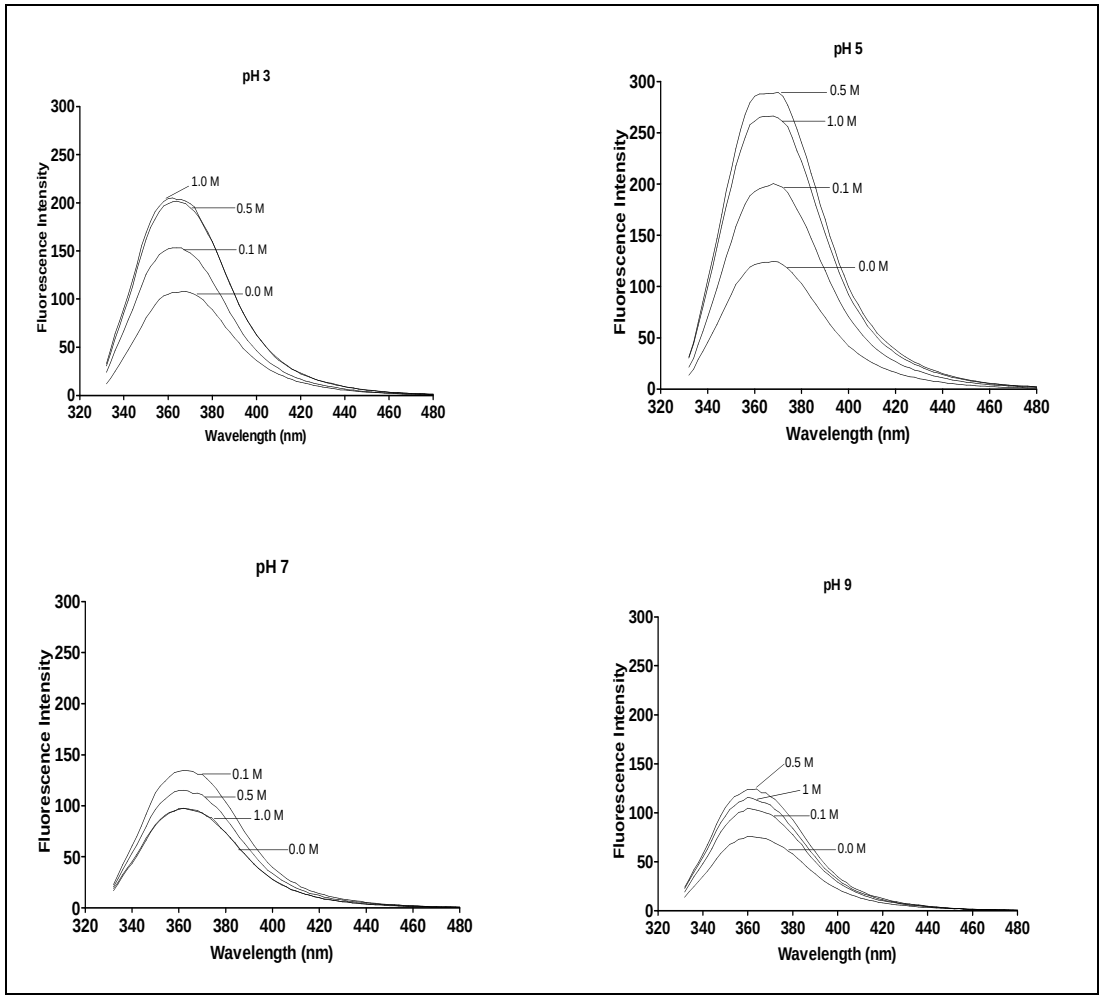


Fig. 3.3 The intrinsic fluorescence spectra of kidney bean vicilin excited at 275 nm (tyrosine+tryptophan) as affected by pH and salt concentration.

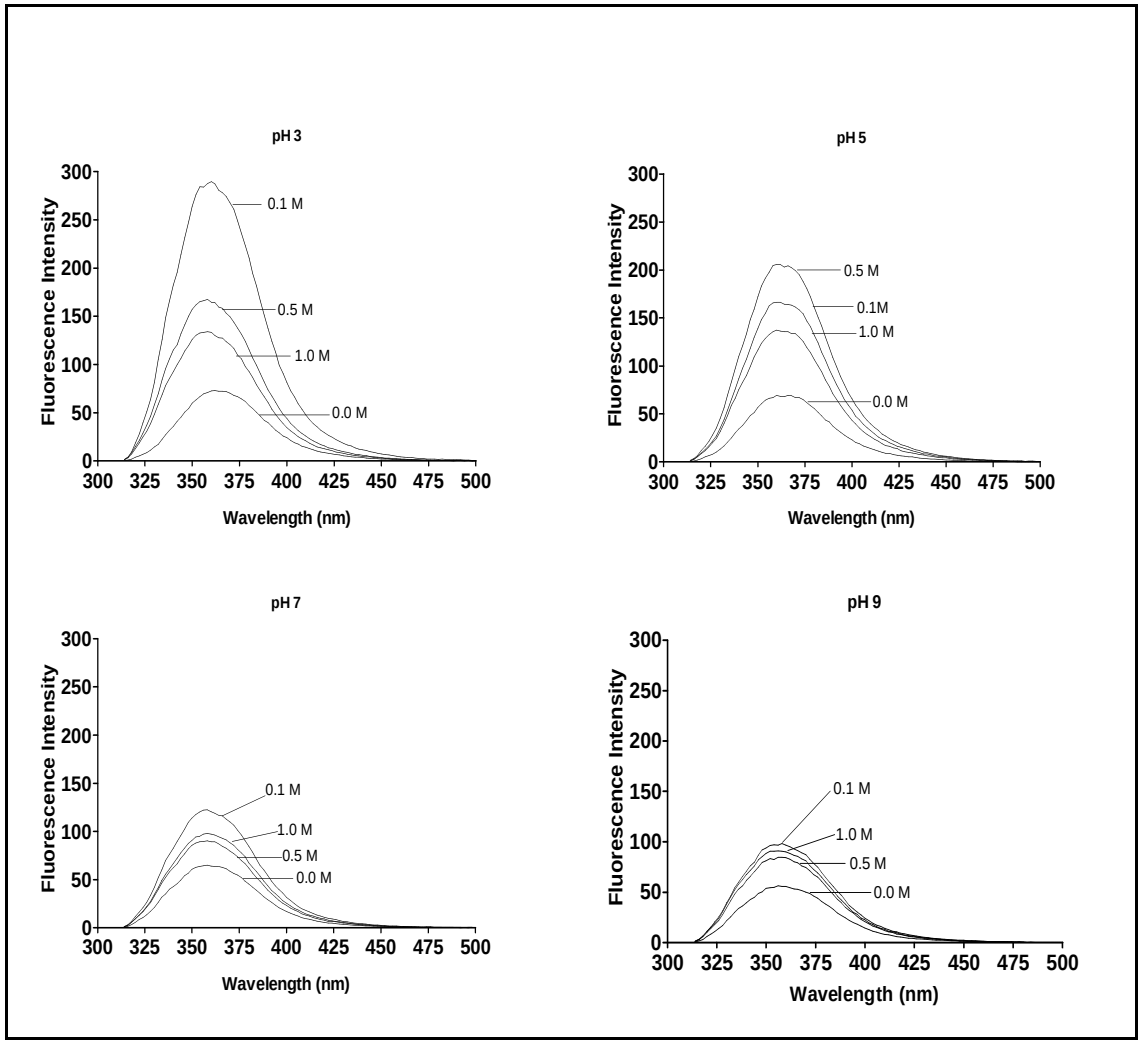


Fig. 3.4 Effect of pH and salt concentrations on the tertiary structure (near-UV CD spectra) of purified kidney bean vicilin.

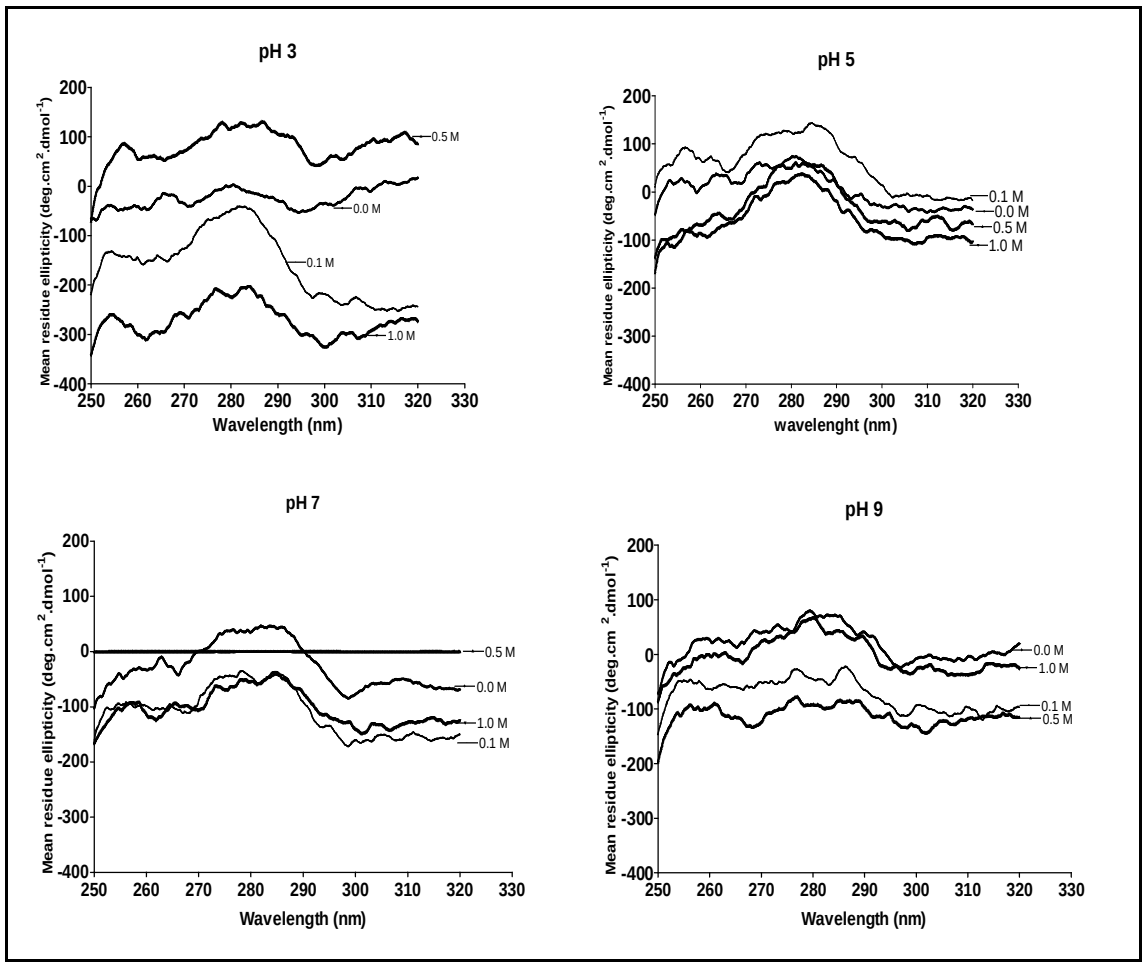
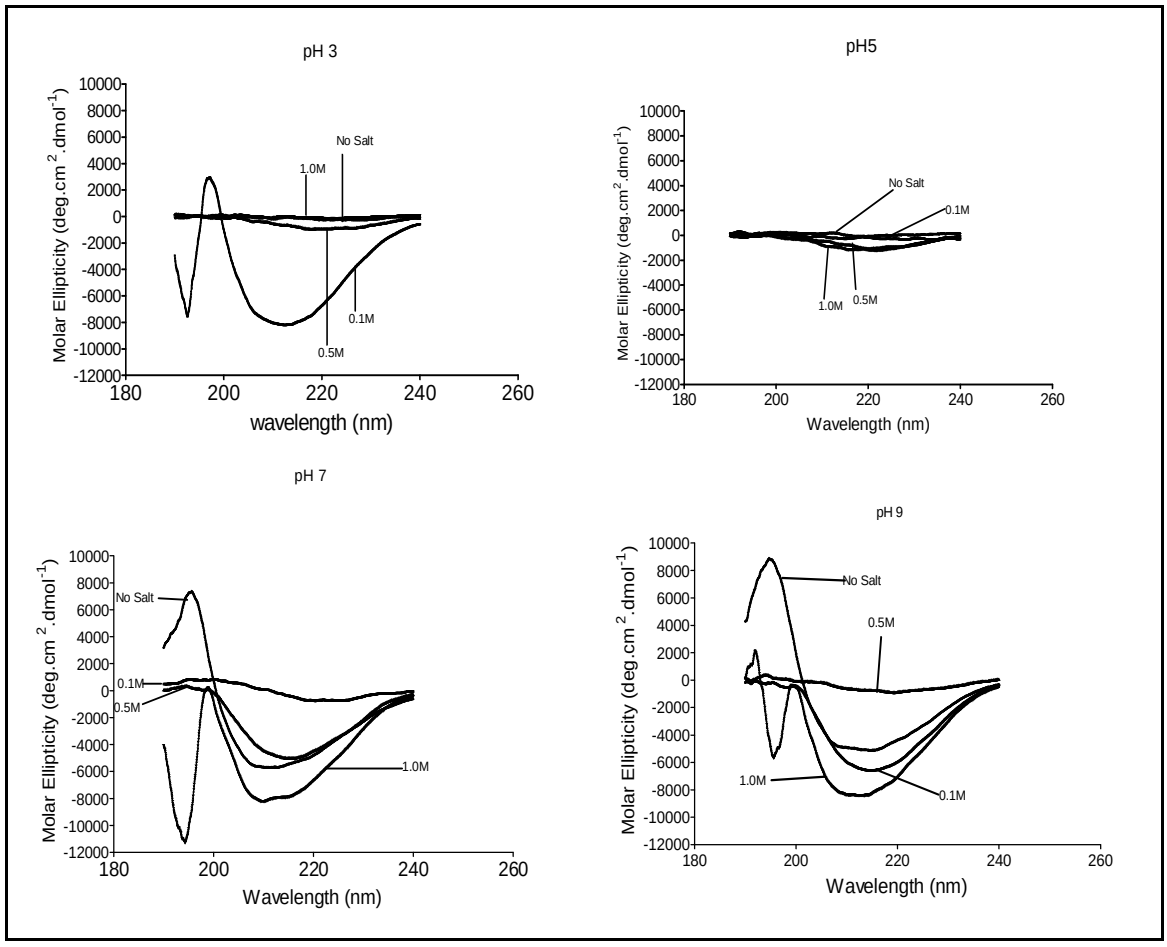


Fig. 3.5 Effect of pH and salt concentrations on the secondary structure (far-UV CD spectra) of purified kidney bean vicilin.



References

Aluko, R. E. (2004). The extraction and purification of proteins: An introduction. In R. Y. Yada (Ed.), *Proteins in food processing* ((pp. 323-346).). Cambridge; Boca Raton: Woodhead Pub; CRC Press.

Aluko, R. E., & McIntosh, T. (2004). Electrophoretic and functional properties of mustard seed meals and protein concentrates. *JAOCS, Journal of the American Oil Chemists' Society, 81*, 679-683.

Arntfield, S.D., Ismond, M.A.H., & Murray, E.D. (1987). Use of intrinsic fluorescence to follow the denaturation of vicilin, a storage protein from Vicia faba. *International Journal of Protein and Peptide Research, 29*, 9-20.

Aykroyd, W.R. and Doughty, J. (1982). Legumes in human nutrition. Food and Agriculture Organization of the United Nations. FAO Food Nutr. Pap. *20*, 1-152.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984). Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography - Biomedical Applications, 336*, 93-104.

Bollini, R., & Chrispeels, M. J. (1978). Characterization and subcellular localization of vicilin and phytohemagglutinin, the two major reserve proteins of phaseolus vulgaris L. *Planta, 142*, 291-298.

Chandra, N. R., Kumar, N., Jeyakani, J., Singh, D. D., Gowda, S. B., & Prathima, M. N. (2006). Lectind: A plant lectin database. *Glycobiology, 16*, 938-946.

Dyer, J. M., Nelson, J. W., & Murai, N. (1992). Biophysical analysis of phaseolin denaturation induced by urea, guanidinium chloride, pH, and temperature. *Journal of Protein Chemistry, 11*, 281-288.

Eskin, N. A. M., & Tamir, S. (2006). *Dictionary of nutraceuticals and functional foods*. Boca Raton, FL: Taylor & Francis Group/CRC Press.

Franzese T.A. (2000). Medical nutrition therapy for rheumatic disorders. In: Krause's Food, Nutrition & Diet Therapy. 10th ed. Mahan L.K., Escortt-Stump S. (eds). W.B. Saunders. Philadelphia. pp 970-986.

Gehrke, C. W., Wall Sr., L. L., & Absheer, J. S. (1985). Sample preparation for chromatography of amino acids: Acid hydrolysis of proteins. *Journal of the Association of Official Analytical Chemists, 68*, 811-821.

Gorinstein, S., Goshev, I., Moncheva, S., Zemser, M., Weisz, M., Caspi, A., Libman, I.; Lerner, H.T.; Trakhtenberg, S. Martín-Belloso, O. (2000). Intrinsic tryptophan fluorescence of human serum proteins and related conformational changes. *Protein Journal, 19*, 637-642.

Hou, Y., Hou, Y., Yanyan, L., Qin, G., & Li, J. (2010). Extraction and purification of a lectin from red kidney bean and preliminary immune function studies of the lectin and four chinese herbal polysaccharides**.** *Journal of Biomedical Biotechnology.*

Panel on Dietary Reference Intakes for Electrolytes and Water, & National Academies Press (USA). (2005). *DRI, dietary reference intakes for water, potassium, sodium, chloride, and sulfate.* Retrieved from http://www.nap.edu/catalog.php?record_id=10925; Materials specified: National Academies Press http://www.nap.edu/catalog.php?record_id=10925

Ishino, K., & Ortega, M. L. (1975). Fractionation and characterization of major reserve proteins from seeds of phaseolus vulgaris. *Journal of Agricultural and Food Chemistry, 23*, 529-533.

Jaffé, W. G., & Hannig, K. (1965). Fractionation of proteins from kidney beans (phaseolus vulgaris). *Archives of Biochemistry and Biophysics, 109*, 80-91.

Joubert, F. J. (1957). Ultracentrifuge studies on seed proteins of the family leguminosae. *J. South African Chem. Inst., 10*, 16-20.

Landry, J., & Delhaye, S. (1992). Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. *Journal of Agricultural and Food Chemistry, 40*, 776-779.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978). A modification of the lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry, 87*, 206-210.

Osborne, T. B. (1894). The Proteids of the Kidney Bean. *Journal of the American Chemical Society, 16*, 633-643.

Wanasundara, P.K.J.P.D. and Shahidi, F.(2003). Flaxseed proteins. In *Flaxseed in human nutrition, second edition*. AOCS Press: Urbana, USA, 2003; pp. 387-403.

Pain, R. H. (1996). Determining the fluorescence spectrum of a protein. In *Current protocols in protein science* (pp. 7.7.1-7.7.19) John Wiley & Sons, Inc.

Pusztai, A., & Watt, W. B. (1970). Glycoprotein II. the isolation and characterization of a major antigenic and non-haemagglutinating glycoprotein from phaseolus vulgaris. *Biochim Biophys Acta, 207*, 413-431.

Sathe, S. K. (2002). Dry bean protein functionality. *Critical Reviews in Biotechnology, 22*, 175-223.

Shi, J., Xue, S. J., Kakuda, Y., Ilic, S., & Kim, D. (2007). Isolation and characterization of lectins from kidney beans (*Phaseolus vulgaris*). *Process Biochemistry, 42*, 1436-1442.

Shimelis, E. A., & Rakshit, S. K. (2007). Effect of processing on antinutrients and in vitro protein digestibility of kidney bean (*Phaseolus vulgaris* L.) varieties grown in East Africa. *Food Chemistry, 103*, 161-172.

Schmid, F.X. Spectra methods of characterizing protein conformation and conformational changes. In *Protein structure: a practical approach*; T. E. Creighton, Ed.; IRL Press: Oxford, 1989; pp. 251-285.

Smriga, M., Ando, T., Akutsu, M., Furukawa, Y., Miwa, K., & Morinaga, Y. (2007). Oral treatment with L-lysine and L-arginine reduces anxiety and basal cortisol levels in healthy humans. *Biomedical Research, 28*, 85-90.

Stanciuc, N., Rapeanu, G., Bahrim, G., & Aprodu, I. (2012). pH and heat-induced structural changes of bovine apo-α-lactalbumin. *Food Chemistry, 131*, 956-963.

Sun, S. M., & Hall, T. C. (1975). Solubility characteristics of globulins from phaseolus seeds in regard to their isolation and characterization. *Journal of Agricultural and Food Chemistry, 23*, 184-189.

Tang, C., & Ma, C. (2009). Heat-induced modifications in the functional and structural properties of vicilin-rich protein isolate from kidney (*Phaseolus vulgaris* L.) bean. *Food Chemistry, 115*, 859-866.

Tang, C., Sun, X., & Foegeding, E. A. (2011). Modulation of physicochemical and conformational properties of kidney bean vicilin (phaseolin) by glycation with

glucose: Implications for structure-function relationships of legume vicilins. *Journal of Agricultural and Food Chemistry, 59*, 10114-10123.

Vaintraub, I. A., Bassüner, R., & Shutov, A. D. (1976). The action of trypsin and chymotrypsin on the reserve proteins of some leguminous seeds. *Food / Nahrung, 20*, 763-771.

Vijayan, M., & Chandra, N. (1999). Lectins. *Current Opinion in Structural Biology, 9*, 707-714.

Waterman, H. C., Johns, C. O., & Jones, D. B. (1923). Conphaseolin: A new globulin from the navy bean, *phaseolus vulgari*. *J. Biol. Chem, 55*, 93-104.

Weisdorf, S. A. (1998). Nutrition in liver disease. In E. Lebenthal (Ed.), *Textbook of gastroenterology and nutrition in infancy* (2nd edn ed., pp. 665-675). Raven Press, New York.: Raven Press, New.

Yin, S-W., Tang, C-H., Wen, Q-B., & Yang, X-Q. (2010a). Conformational and thermal properties of phaseolin , the major storage protein of red kidney bean (phaseolus vulgaris L.). *Journal of the Science of Food and Agriculture, 91*, 94-99.

Yin, S-W., Tang, C-H., Wen, Q-B., Yang, X-Q., & Yuan, D-B. (2010b). The relationships between physicochemical properties and conformational features of succinylated and acetylated kidney bean (phaseolus vulgaris L.) protein isolates. *Food Research International, 43*, 730-738.

Yin, S-W., Huang, K-L., Tang, C-H., Yang, X-Q., Wen, Q-B., & Qi, J-R. (2011a). Surface charge and conformational properties of phaseolin, the major globulin in red kidney bean (*Phaseolus vulgaris* L.): Effect of pH. *International Journal of Food Science and Technology, 46*, 1628-1635.

Yin, S-W., Tang, C-H., Yang, X-Q., Wen, Q-B., (2011b). Conformational study of red kidney bean (Phaseolus vulgaris L.) protein isolate (KPI) by tryptophan fluorescence and differential scanning calorimetry. *Journal of Agricultural and Food Chemistry, 59*, 241-248.

CHAPTER 4

EMULSION, FOAM AND THERMAL PROPERTIES OF PURIFIED KIDNEY BEAN VICILIN

S. Mundi, R.E. Aluko*

The Department of Human Nutritional Sciences and the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

*Corresponding author: Rotimi E. Aluko, Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, MB R3T 2N2, Canada; E-mail: alukor@cc.umanitoba.ca, Tel: +1(204) 474-9555, Fax: +1(204) 474-7593

To be submitted

4.0. Abstract

Kidney bean vicilin prepared by ammonium sulphate precipitation was purified by fast protein liquid chromatography. The purified vicilin was analyzed for its foaming and emulsifying properties. Also, the thermal stability of the vicilin protein was monitored by far- and near-UV circular dichroism (CD) and differential scanning calorimetry (DSC) experiment. The surface diameter ($d_{3,2}$) was reduced at higher ionic strength and high protein concentration, implying better emulsifying capacities. Both DSC, and far-and near-UV CD data provided valuable insights into the thermal folding/unfolding transitions of kidney bean vicilin protein. The DSC showed the highest T_m and ΔH at pH 5, decreasing at acidic and alkaline pH, implying a more stable protein at the isoelectric point of the protein. The T_m of the native protein at neutral pH was 90.31. The β-sheet content increased with increasing temperature until 60^oC when a steady decrease was recorded, with some retention of β-sheet even at 80^oC.

Key words

Kidney bean vicilin, foaming, emulsion, far- and near-UV circular dichroism, differential scanning calorimetry

4.1. Introduction

The ability of proteins to participate in emulsion and foam formation as well as to stabilize created emulsions and foams are critical characteristics for the development of traditional or novel food products (Cabra *et al.* 2008). Proteins are the most common class of foaming and emulsifying agents in the food industry, and the stability of these very vital functional properties (emulsification and foaming) during processing, storage and handling is very important to guarantee the commercial success of various products that depend on them (Salvador *et al.* 2010). As surfactants in reducing interfacial tension, proteins have the advantages of being naturally occurring ingredients, cheap and widely available, thus making them ideal ingredients (Wilde *et al.,* 2004). Both emulsification and foaming behaviours of proteins are affected by their structural conformations. This is because the tertiary or quaternary structures of polypeptides have to unfold and spread to cover the surface area where they form an interfacial membrane by adsorbing to the oil/water or air/water interface (Townsend and Nakai, 1983). The unfolding events during structural conformation changes are influenced by thermal properties of proteins and the predominant environmental conditions, such as pH and ionic strength of the medium, as well as molecular flexibility, dielectric constant, and the presence of other molecules including air, fat, and denaturants (Townsend and Nakai 1983; Yin *et al.* 2010). The protein will assume different conformations as environmental conditions change and therefore, will interact differently with other components within the food system. Thus, the thermal stability of food proteins is necessary for structural conformations that contribute to emulsion and foam stability (German *et al.* 1982). Since temperature is a

very important factor in a process operation of food product development (as high temperature causes protein unfolding and loss of functionality) (Leon *et al.* 2003), emulsification, foaming and thermal characteristics of proteins require significant attention. In addition, the physiological significance of biologically active proteins, usually closely related to the structural properties, is also affected by changes in environmental factors.

Large differences in the ability to form and stabilize dispersed systems have been observed among different food proteins (Gauthier *et al.* 1993). Milk and egg proteins demonstrated excellent emulsification and foaming properties, but the food industry is actually looking for cheaper protein sources with similar functionality (Salvador *et al.* 2010). Kidney bean flour has been reported to have excellent functional properties, e.g., gelation capacity, emulsifying activity and emulsion stability, when compared to soybean flour (Chau and Cheung, 1998), which increases potential utilization of kidney beans in food formulations. Generally the functionalities of seed flours can be attributed to the proteins contained in them. Phaseolin, the major storage protein in kidney bean seeds showed pH-dependent association–dissociation behavior (Yin *et al.* 2010), which may affect all or some of its functional properties. The emulsification capacity (EC), emulsion stability (ES), foaming capacity (FC), and foam stability (FS) vary with the source of protein, its concentration, pH, ionic strength (the type of salt and its concentration), preparation method and thermal properties of the protein (Sathe, 2002). However, there is the need to expand current knowledge on how heat treatment combined with the effects of the variation in ionic strength and pH may affect emulsification and foaming properties of purified kidney bean globulin. In the present study therefore, we have

examined the effects of variations in NaCl concentration and shifts in pH on emulsion and foaming properties of purified vicilin. We have also examined pH and temperature-dependent unfolding of the vicilin protein in the presence or absence of NaCl using differential scanning calorimetry analysis (DSC) and circular dichroism (CD). Results from this study could improve our understanding of how kidney bean proteins will respond to changes in heat, pH and salt concentrations, which are usually encountered during industrial manufacture of food products.

4.2. Materials and Methods

4.2.1 Preparation of kidney bean globulin

Kidney bean seeds purchased from a local store in Winnipeg, Manitoba were cleaned and milled into powder using a Retsch ZM200 centrifuge mill (Retsch GmbH, Haan, Germany). The powder (10% w/v) of was dispersed in 0.1 M phosphate buffer containing 0.4 M NaCl and stirred for 2 h at 4^{o}C. The supernatant from centrifugation (9000 × *g*, 45 min at 4^{o}C) was saturated with 40% solid ammonium sulfate at 4^{o}C. After stirring for 2 h, the slurry was centrifuged (9000 × *g*, 45 min at 4^{o}C) and the precipitate discarded. The supernatant was further adjusted to 80% saturation with addition of solid ammonium sulphate, stirred, and centrifuged as before. The precipitate was collected, dispersed in water and then dialyzed against water using dialysis membrane of 6-8 kDa molecular weight cut off. The contents of the dialysis bag was then centrifuged (9000 × *g*, 45 min at 4^{o}C) to obtain globulin (precipitate), freeze dried and used for subsequent analyses after the protein concentration was determined using the modified Lowry method (Markwell *et al.* 1978).

4.2.2 Purification of vicilin by fast protein liquid chromatography (FPLC)

The kidney beans globulin protein was separated using ÄKTAPurifier FPLC system equipped with a size exclusion column, HiLoad 26/60 Superdex 200 Prep grade (GE Healthcare, Montreal, PQ). Sodium phosphate (0.1 M) prepared to contain 0.4 M NaCl was used as the equilibration buffer for the column, and as an elution buffer. Two millilitres of 100 mg/mL globulin protein solution, filtered through 0.2 μm filter disks was loaded onto the Superdex column and run at a flow rate of 2.5 mL/min; eluted proteins were detected from the UV absorbance at 214 nm. The major fraction was collected and desalted using Hiprep 26/10 desalting column, following which the sample was freeze dried, analyzed for protein content (Markwell, Haas, Bieber, & Tolbert, 1978) and stored at −20 °C until further analysis.

4.2.3. Foaming capacity and stability

Foaming capacity (FC) and foaming stability (FS) of the samples was studied according to the method previously described (Aluko *et al.* 2009) using slurries that were prepared by dispersing 0.1-0.3g samples in 50 mL graduated centrifuge tubes containing 5 mL 0.1 M phosphate buffer, pH 3.0, 5.0, 7.0, and 9.0. Sample slurries were homogenized at 20,000 rpm for 1 min using a 20 mm foaming shaft on the Polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland). The capacity of the continuous phase to include air (foam capacity) using the mean of three measurements was determined thus;

$$\text{Foam Capacity (FC)} = \frac{\text{Volume after homogenization-Volume before homogenization}}{\text{Volume before homogenization}} \times 100$$

The ability to retain air (foam stability) was calculated by measuring the foam volume after storage at room temperature for 60 min and expressed as percentage of the original foam volume.

4.2.4 Emulsifying capacity and emulsion stability

A series of emulsions were prepared in 50 mL plastic centrifuge tubes, by adding 1 ml of pure commercial canola oil to 25, 50 and 75 mg purified kidney bean vicilin protein dispersed in 5 ml 0.01 M phosphate buffer prepared at varying pH (3, 5, 7 and 9) and NaCl concentrations (0.0, 0.1, 0.5 and 1 M). The protein solution was then homogenized at 22,000 rpm for 1 min using a Polytron PT 10-35 homogeniser equipped with a 20-mm non-foaming generator. The emulsifying capacity of the protein sample was determined by measuring the particle size distribution and mean particle diameter as previously described (Aluko *et al.,* 2009). The mean oil droplet size ($d_{3,2}$) and volume weighted mean ($d_{4,3}$) of the emulsions were determined in a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK) with Milli-Q water as dispersant. Emulsions were prepared in duplicate samples with three Mastersizer measurements per sample; making results means of six determinations. Emulsions were kept at room temperature for 1 h without agitation and the particle size distribution and mean particle diameter were measured again to assess stability.

4.2.5. Circular dichroism (CD)

CD measurement was performed at five different temperatures (40, 50, 60, 70 and 80^{o}C) using JASCO J-815 CD spectropolarimeter equipped with JULABO F25-ME circulating water bath for the regulation of the cell holder temperature. Conformational changes in

secondary and tertiary protein structure were monitored in the spectral range 190-240 nm and 250-320 nm, respectively. For secondary structure determinations, the cuvette with pathlength of 0.05 cm was used, and scanning conditions were as follows: sensitivity 2 mdeg/cm; time constant 1.0 s; scan speed 5 nm/min; wavelength expansion 5 nm/cm. The near-UV region of the spectrum was measured in a 0.1 cm cuvette under similar experimental conditions and instrument settings as the far-UV, except the difference in wavelength. All samples were centrifuged at 10,000x*g* for 10 min prior to analysis and the CD spectra were obtained as the average of three consecutive measurements with automatic subtraction of the buffer spectra. After subtracting appropriate blanks, mean residue ellipticity was calculated, using:

$[\theta] = \theta_{obs}$ x MRW/10cl

Where, θ_{obs} is the measured ellipticity in degrees, MRW is the mean residue weight, c is the concentration of protein in g/ml and l is the path length in cm (Sundd *et al.,* 2004). A mean residue molecular weight of 110 was used.

4.2.6. Differential scanning calorimetry (DSC)

The thermal transitions of the purified kidney bean vicilin were monitored at varying pH and salt concentrations according to the procedure of Meng and Ma (2002) with some modifications. Briefly, 2.0 mg of samples were weighed into aluminum pans, and 10 µl 50 mM phosphate buffer (pH 3, 5, 7 and 9) prepared to contain 0.0, 0.1, 0.5 and 1M NaCl was added. The pans were hermetically sealed and heated in the calorimeter from 25 to 120^{o}C at a rate of 10^{o}C/min, with a sealed empty pan used as a reference. DSC experiments were performed on a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, DE). Peak or denaturation temperature (T_d), enthalpy change of denaturation (ΔH)

was computed from the thermograms by the universal analyzer 2000, version 4.1D (TA Instrument-Waters LLC, USA).

4.3. Result and Discussion

4.3.1. Foaming properties

The influence of protein concentration (0.1, 0.2, and 0.3 g in 5 mL) and pH (3, 5, 7, and 9) on FC and FS of kidney bean vicilin is showed in Figures 4.1A and B. The ability of kidney bean vicilin protein film to adsorb to the air/water interface as a result of their amphiphilic nature increased with increasing protein concentration (Fig. 4.1A). All the samples revealed increased foam forming ability with the increase in concentration of samples, especially between 0.1-0.2 g, but with a decrease in capacity between 0.2-0.3 g concentrations, probably due to the interfacial film around the air/water becoming saturated by the protein. Whether or not good foams can be produced may be dependent on the amount of protein present that can unfold to form a strong interfacial membrane and adsorb to the air/water interface. A similar pattern was reported by Salvador *et al.* (2010) for porcine red cell protein concentrate and by Martin et al. (2002) for a series of flexible to rigid/globular proteins: *β*-casein, *β*-lactoglobulin, ovalbumin, and (soy) glycinin. Fig. 4.1A also showed the dependence of the minimum concentration required to reach highest FC on the pH of the solution. The minimum FC was obtained close to the isoelectric point (pH 5) of the vicilin protein while the highest FC was at the alkaline pH (pH 9). It is not surprising that the least FC was observed at pH 5, which is the point of least protein solubility, since solubility is a prerequisite for good foaming properties. Ragab *et al.* (2004) and Lawal *et al.* (2005) also observed pH-dependent foaming

characteristics in cowpea protein isolate and FC of albumin and globulin of African locust bean, respectively.

Foam stability of the vicilin foams was also both protein concentration and pH dependent as shown in Fig. 4.1B. At lower protein concentrations and at isoelectric pH of the protein sample, the foams drained very fast, with a gradual increase at higher protein concentrations and acidic and alkaline pH. The observed decreased stability of foams at lower protein concentration is consistent with the conclusion of Martin *et al.* (2002) who showed that foam drainage was dependent on initial amount of foam formed. Generally, foam stability requires the formation of a cohesive, viscous, elastic, continuous, air-impermeable film around each gas bubble, and pH and temperature are crucial factors for foam stability (Sai-Ut *et al.* 2009). Therefore, the higher foam stability reported at higher concentrations in the current study suggests that more proteins were available for greater protein-protein interactions to form strong interfacial membranes at the air-water interface. Also at higher protein concentrations of 0.2 and 0.3 g, the stronger interfacial membranes were more resistant to coalescence than membranes formed at the 0.1 g protein concentration. Compared to the 25% FC observed for the 0.1g in 5 mL concentration in the current study, Siddiq *et al*. (2010) reported a higher value (45.7%) for the same concentration of kidney bean flour. The reason for the higher foaming capacity for the flour may be attributed to the higher carbohydrate content of the flour which must have been removed during the purification procedures used in the current study. Adebowale and Lawal (2003) reported increased foaming capacity with increase carbohydrates content. DiLollo (1993) observed 40% FC for white kidney bean protein isolate with 72.8% protein content but the author observed 21% FC for the isolate with

95.7% protein content. This result demonstrated that, increased protein content which can be obtained through protein purification may reduce the capacity for foaming, probably due to increased protein-protein interaction and therefore reduced ability of the proteins to unfold at the air-water interface, which limits capacity to encapsulate air bubbles.

4.3.2. Emulsion Properties

The amphiphilic nature of protein molecules facilitates adsorption onto the oil/water interface in such a way that nonpolar (hydrophobic) patches are oriented towards and makes contact with the oil phase while hydrophilic groups interact with the aqueous phase. Fig. 4.2 shows the effect of protein concentration and pH on emulsion capacity of the vicilin protein. At each of the tested pH and ionic strength, oil droplet size ($d_{3,2}$) showed consistent reduction as protein concentration was increased, which indicates increased emulsion forming capacity as more protein molecules become available at the oil/water interface. This trend has been reported previously for pea seed flour fractions (Aluko *et al.,* 2009). The emulsion was also markedly affected by pH. Irrespective of the ionic strength, most of the emulsions had least oil droplet sizes and hence better ability to form emulsions at pH 3. For most of the emulsions and within each protein concentration, the oil droplet size was higher at pH 9.0, which is similar to previous reports (Aluko and Yada 1995; Hayakawa and Nakai 1985; Kato and Nakai 1980). The poor emulsion forming ability of vicilin at pH 9.0 could be explained in terms of protein surface hydrophobicity which is an important phenomenon in emulsion formation. The pH of solutions influence their emulsification properties primarily by altering the charge on protein molecules (Halling 1981), based on the hydrophilic-lipophilic balance. At acidic pH, increased protonation of carboxyl and hydroxyl groups will reduce electrostatic

attractions, which favour increased protein-protein interactions to form strong interfacial membranes. Conversely, at the alkaline region there is increased deprotonation (ionization) of ionizable amino acid side chains, resulting in high electrostatic repulsions, which reduces ability of the proteins to form interfacial membrane during emulsion formation (Aluko and McIntosh 2001; Salvador *et al.* 2010). The surface diameter ($d_{3,2}$) was reduced as ionic strength increased, probably as a result of increased protein solubility and the charged environment reduces coalescing ability of the formed oil droplets to maintain small oil droplet sizes. The results showing positive effects of NaCl on emulsification ability of vicilin is consistent with previous results reported for soybean 7S protein (Fukuda *et al.* 2005), and cowpea proteins (Aluko and Yada 1995; Zhang *et al.,* 2009a) and chickpea proteins (Zhang *et al.* 2009b).

As seen in Fig. 4.3, protein concentration also had a positive influence on the emulsion stability, probably by increasing viscosity of the continuous phase and minimizing the effects of density differences between both phases of the system (Makri and Doxastakis, 2006). A high protein concentration would have enhanced formation of thicker interfacial membranes that can better resist oil droplet coalescence when compared to a low protein concentration. However, at 0.5 and 1.0 M NaCl concentrations, the influence of protein concentration on emulsion stability was minimized. Fig 4.3 also showed that unlike protein concentrate, pH variations had minimal effects on emulsion stability at each concentration of NaCl. However, emulsion stability slightly decreased at pH 7 and 9 with increase in NaCl concentration, suggesting weak interfacial membrane formation as protein-protein interactions are reduced due to increase in net molecular charge. Generally, the vicilin-stabilized emulsions were very

stable (>60%) at all pH values and NaCl concentrations tested. This may be attributable to the presence of relatively small oil droplets (<10 μm) in the vicilin-stabilized emulsions.

4.3.3. Circular dichroism (CD) spectroscopy

CD spectroscopy can be used to monitor changes in protein folding and changes in conformations. The loss of CD signals at extreme pH, high ionic strength or on addition of denaturing agents (such as urea or guanidinium chloride), or even by an increase in temperature can be used to provide quantitative estimates of the stability of the folded state of the native protein (Kelly and Price 2006; Kelly *et al.* 2005). Figures 4.4 and 4.5 shows respectively, the far- and near-UV CD spectra of purified kidney bean vicilin at five different temperatures (40, 50, 60, 70 and 80^{o}C).

4.3.3.1. Far-UV CD Spectra: Secondary structure of the purified kidney bean vicilin was investigated using far-UV (190-240 nm) CD measurements. Generally, for the unheated samples no transitions were observed at acidic pH 3 and 5 (Fig. 4.4). However positive signals, around 195 nm, typical of β-sheet were observed for samples at pH 7 and 9 but not for pH 8. A negative ellipticity, most probably for higher order secondary structure (α-helix) was also observed around 215 nm for pH 7 to 9. Tang & Sun (2011) and Tang *et al.* (2011) reported similar transitions at pH 7 in the far-UV spectra of kidney bean vicilin purified using DEAE-Sepharose fast flow column chromatography. Since no signals were observed for samples at acidic region, the temperature-dependent secondary structural changes were only investigated at pH 7-9 using far-UV CD spectroscopy. At pH 7, there was a gradual increase in the molar ellipticity of the peaks at 195 nm with increasing temperature from 25 to 60^{o}C, but ellipticity started to decrease as temperature

approached 70°C with a significant loss of signal at 80°C. The initial increase in ellipticity indicates a steady increase in β-sheet content which is the most dominant secondary structure element present in bean seed proteins (Carbonaro, 2006). However, the negative ellipticity (peak at 215 nm) showed a steady loss of signal with increasing temperature, demonstrating a continuous loss of higher order secondary structure of the α-helical content with increase in heating temperature. A similar trend was observed at pH 8 and 9, where the β-sheet increased at lower temperatures (40-60°C), with a steady loss of α-helical content. The loss of helicity could be due to disruption of inter-domain and intra-domain structure at higher temperature as a result of breakage of hydrogen bonds. However, at all the pH values studied there was some minimal retention of α helical content even at 80°C, as shown by the negative transitions at around 215 nm. With the exception of 60 and 80°C at pH 7, Table 4.1 also shows minimal content of helical structure when compared to the other secondary structure fractions. Acharya *et al.* (2002) however reported continuous loss of α-helical content amongst the isoforms of human apolipoprotein E (apoE2, apoE3, and apoE4) with a corresponding increase in the β-sheet at increasing temperatures as high as 90°C. The presence of high content of random secondary structure fraction is also an indication of a highly disordered protein structure arising from heat treatment (Table 4.1). Most of the substantial changes in secondary structure as a result of heat treatment were observed for the β-sheet and random fractions but not the α-helix and β-turn fractions (Table 4.1). Far-UV CD spectra of proteins often show pronounced temperature dependence. The signal response to any small change in temperature often reflects a true conformational change and is not simply due to changes in the optical properties of a helix or strand. The changes, which are often linear with

temperature, are probably due to fraying of the ends of a helix or to changes in helix–helix interactions (Greenfield, 2004; Greenfield, 2007)

4.3.3.2. Near-UV CD Spectra: Near-UV CD Spectra records signals arise from aromatic residues in the region between 260 and 320 nm, thus probing the protein's tertiary structure. The present investigation employs near-UV CD spectroscopy to further understand the effects of varying temperatures on structural conformations of the kidney bean vicilin. Fig. 4.5 shows the near-UV CD Spectra of the purified kidney bean globulin as affected by pH and temperature. As can be seen from the figure, there was a loss of tertiary structure while with temperature increases, indicating temperature-dependent protein denaturation from slightly folded to the unfolded conformations. At all pH measurements, there was a reduction in ellipticity as temperature was increased with 40^{o}C having the highest transition at pH 5, being the isoelectric point of vicilin proteins where the protein may be highly folded. Unlike the far-UV CD spectra and with the exception of 40 and 50^{o}C, the near-UV CD spectrum showed characteristic spectra of a fully unfolded protein i.e., there was no transition at higher temperature. This can be explained by the fact that, in contrast to the far-UV CD which shows signal of a native-like spectrum when proteins are only partially unfolded and the intermediate state still retain some of the native secondary structure, the near-UV CD have a highly fluid tertiary structure and therefore has a near-UV CD spectrum characteristic of a fully denatured form (Martin & Schilstra, 2008).

4.3.4. Differential scanning calorimetry

An understanding of the thermal properties of kidney bean vicilin will enhance its potential utilization as food ingredient, especially in products that involve heat

treatments. In the current DSC study, the effects of buffer conditions including variations in pH and ionic strength were examined on the thermal transition properties of purified kidney bean vicilin. In aqueous solution, there is usually equilibrium between the native and the denatured conformation of proteins. For a protein to unfold, non-covalent intramolecular interactions between amino acid side chains which serve as stabilizing forces need to be broken, and this is usually achieved when the conformational entropy overcomes stabilizing forces (Gill, Moghadam & Ranjbar, 2010). Fig. 4.6 and Table 4.2 show DSC thermograms of the purified kidney bean vicilin and their associated thermal properties, respectively. The peak transition or denaturation temperature (T_d) and the enthalpy change (ΔH) were measured as the area under endothermic peak, at varying pH (3, 5, 7 and 9) and NaCl concentrations (0.0, 0.1, 0.5 or 1.0 M). The DSC thermograms showed single endothermic peak at all pH values and ionic strength used in this study.

4.3.4.1. Effect of pH

When no salt was added to the protein solutions, the T_d of the native protein obtained at the neutral pH was 90.31^{o}C (Table 4.2). The result showed the highest T_d and ΔH at pH 5, that is, near the isoelectric pH (4.5) of the kidney bean vicilin, with both the T_d and ΔH values decreasing at acidic and alkaline pH. Similar trends were observed for the both the T_d and ΔH values when the buffer was adjusted to contain 0.1 and 1 M NaCl. The higher T_d and ΔH at pH 5 imply a more stable protein since proteins which are more stable are less vulnerable to unfolding and precipitation. Most proteins are more stable around their isoelectric pH, where repulsive forces are low, and there are increased protein-protein interactions. At pH lower or higher than the isoelectric point, large net charges are

induced and repulsive forces increase, resulting in unfolding of proteins and decreased T_d values.

4.3.4.2. Effect of salt

The effect of NaCl concentration is shown on Table 4.2, which indicates decreases in ΔH and T_d of the kidney bean vicilin upon addition of 0.1 M salt. The decreases in ΔH and T_d indicate thermal denaturation of the vicilin component, since partially unfolded protein would require lower enthalpy to denature (Meng and Ma, 2002). As the NaCl concentration was increased from 0.1 to 0.5 M, the ΔH increased at almost all the pH tested (except for pH 9) with variations in the T_d values. However, a further increase to 1 M NaCl showed no substantial increases in the T_d values, except at pH 3. This result showed that, addition of NaCl at the concentrations tested helped to stabilize (increased ΔH values) kidney bean vicilin at pH 7 and 9 but not at acidic pH values. Similar observations were reported for red bean globulin (Meng and Ma, 2002) and oat proteins (Ma *et al.* 1988). Meng and Ma (2002) attributed the stabilizing effect of the low NaCl concentration on the globular protein to the induction of hydration of the protein molecules. Hydration or the presence of moisture around proteins increases the amount of heat energy that is required to denature the protein.

4.4. CONCLUSIONS

Far-UV CD spectroscopy indicated that initial heating of the vicilin greatly increased β-sheet content up till 60^{o}C, but showed a steady loss in the tertiary structure as temperature was increased further. The denaturation temperature of vicilin as measured by DSC was 90.31^{o}C. The highest denaturation temperature (T_d) and enthalpy of denaturation (ΔH) was obtained at pH 5, which is close to the isoelectric point (pH 4.5) where protein

structure is highly folded and held together by non-covalent (hydrophobic and hydrogen) interactions.

Table 4.1. Effect of temperature and pH on the secondary structure of purified kidney bean vicilin as measured by circular dichroism spectroscopy

	pH 7.0						pH 8.0						pH 9.0					
Temp (^{o}C)	25	40	50	60	70	80	25	40	50	60	70	80	25	40	50	60	70	80
Helix (%)	7	6	6	34	6	24	1	7	6	3	7	11	6	0	6	2	8	17
β-Sheet (%)	20	30	32	66	31		46	18	24	40	21	7	30	51	32	42	15	10
β-Turns (%)	16	18	18	16	18	20	20	16	17	20	17	16	18	21	18	20	18	20
Random (%)	57	46	45	0	46	40	33	60	53	37	56	66	46	27	45	36	59	54

Table 4.2. Effects of pH, ionic strength and thermal denaturation on the thermal transition properties of the FPLC purified kidney bean vicilin.

pH	Enthalpy (ΔH) (J/g)				Td (Denaturation temperature, ^{o}C)			
	Control	**0.1M**	**0.5 M**	**1 M**	**Control**	**0.1 M**	**0.5 M**	**1 M**
3	13.99	12.72	12.90	12.30	91.02	89.79	91.71	90.80
5	16.35	13.38	15.36	11.90	91.66	90.02	89.63	93.11
7	10.06	11.85	12.89	12.62	90.31	88.65	88.76	89.53
9	5.90	10.61	13.45	13.57	89.02	90.44	88.88	89.64

Fig. 4.1: Effects of pH and sample concentration (g/ml) on foaming capacity (A) and foaming stability (B) of fast protein liquid chromatography purified kidney bean vicilin. For each plot, bars with different letters are significantly different (P≤0.05)

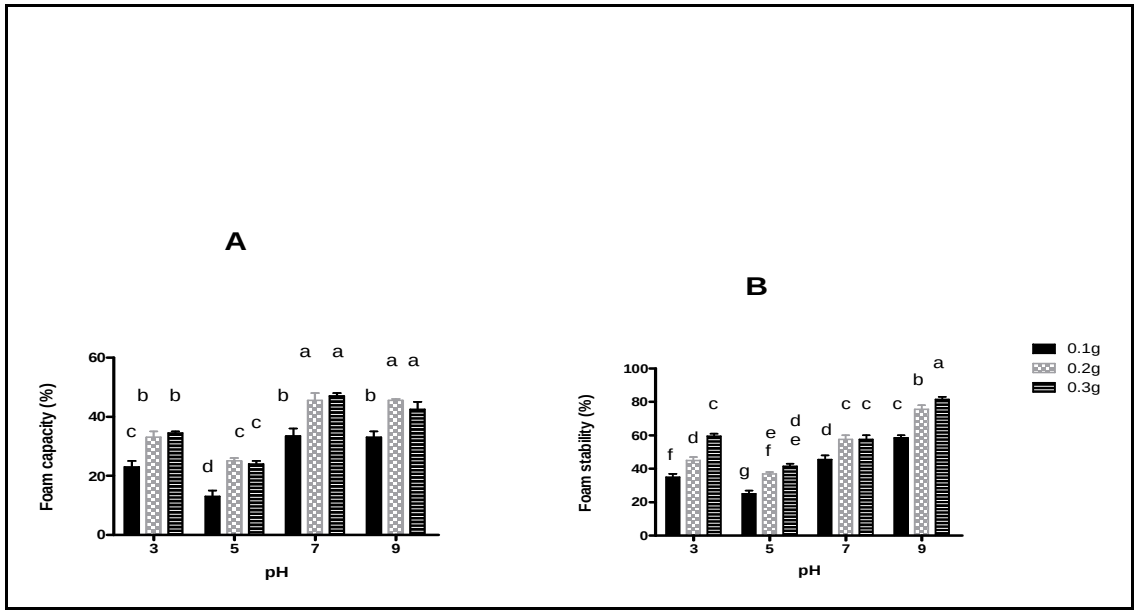


Fig. 4.2: Effect of pH, NaCl and sample concentration (mg/ml) on the oil droplet size ($d_{3,2}$) of oil-in-water emulsions stabilized kidney vicilin fraction. For each plot, bars with different letters are significantly different (P≤0.05

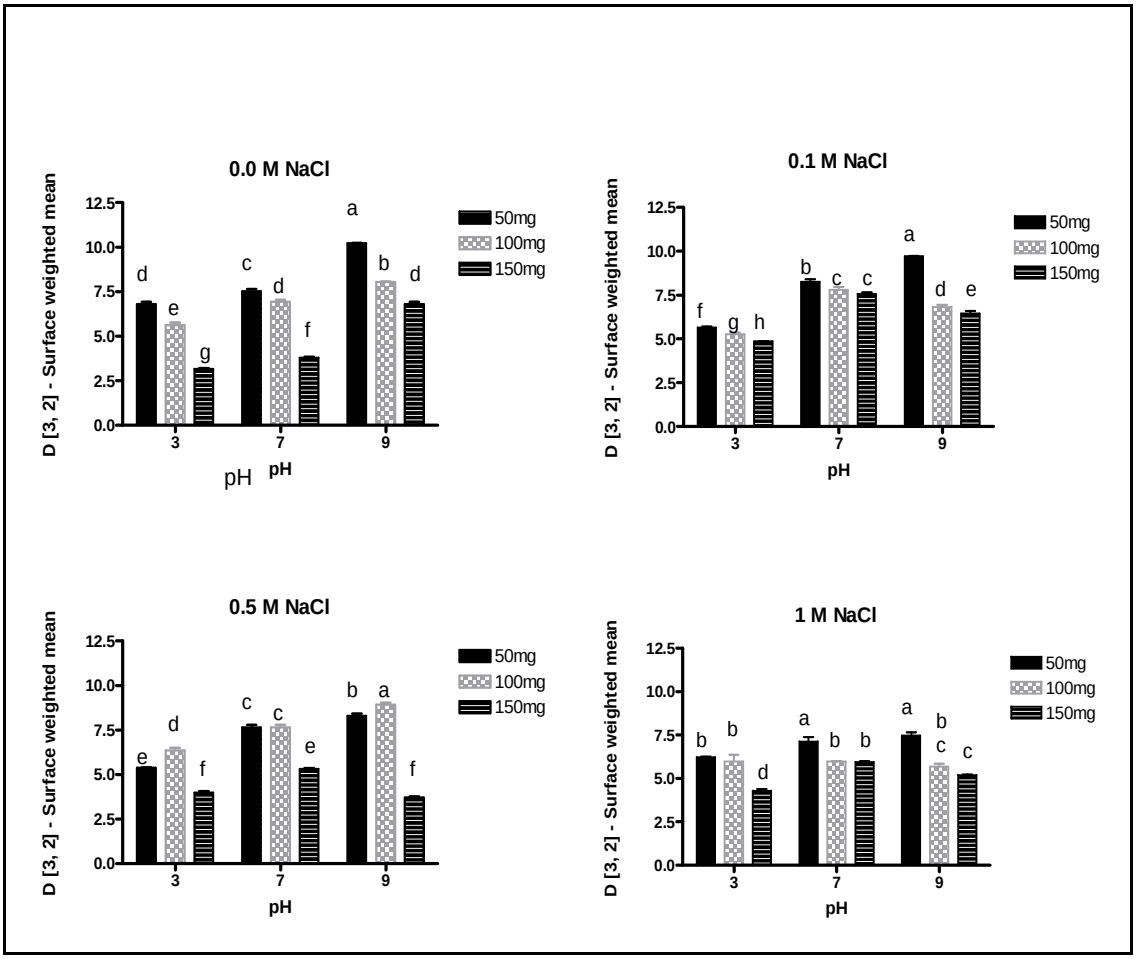


Fig. 4.3: Effects of pH and flour concentration (mg/ml) on emulsion stability (percent increase in oil droplet size) made with kidney bean vicilin. For each plot, bars with different letters are significantly different ($P \leq 0.05$)

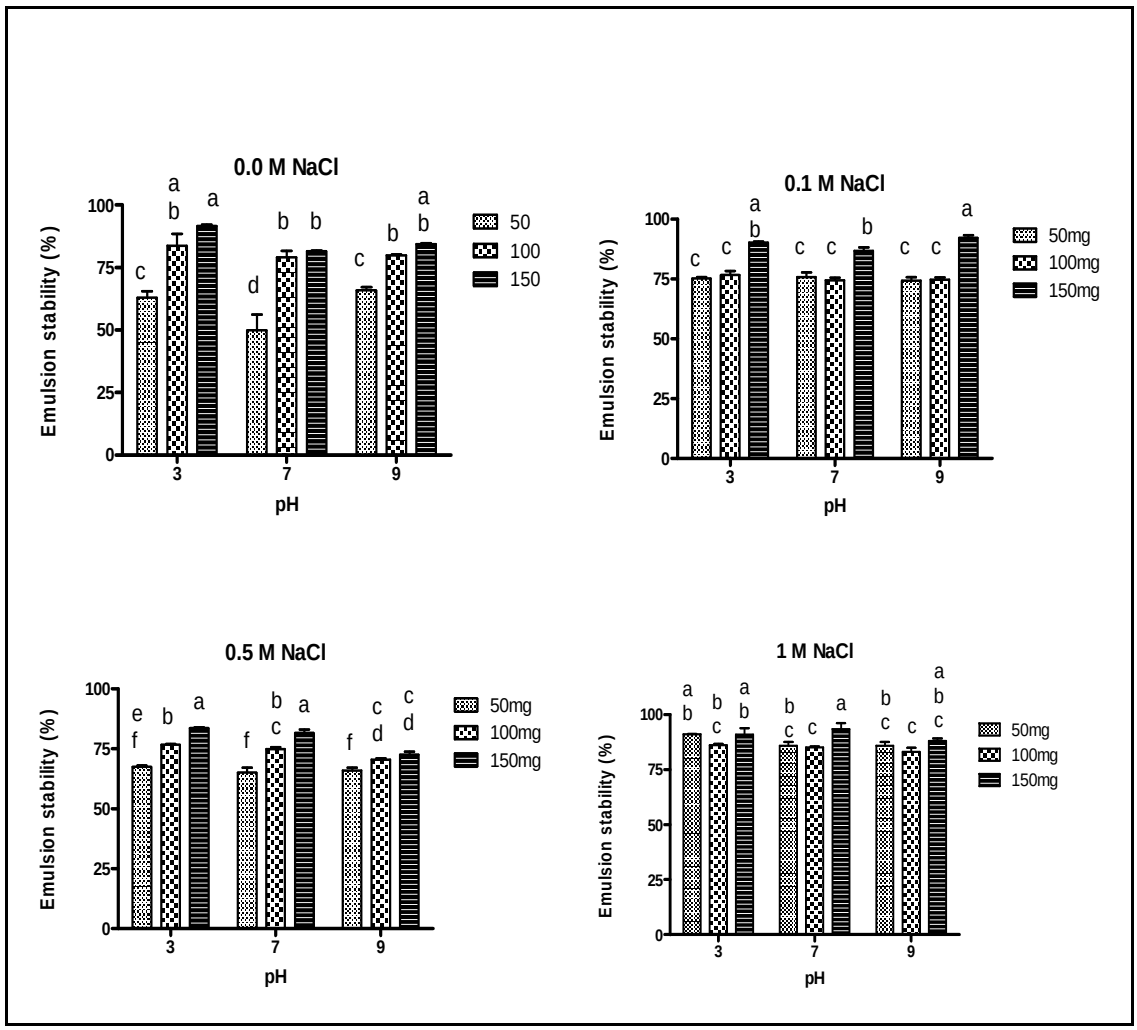


Fig 4.4: Effect of heat and pH on the far-ultraviolet circular dichorism spectra of kidney bean vicilin.

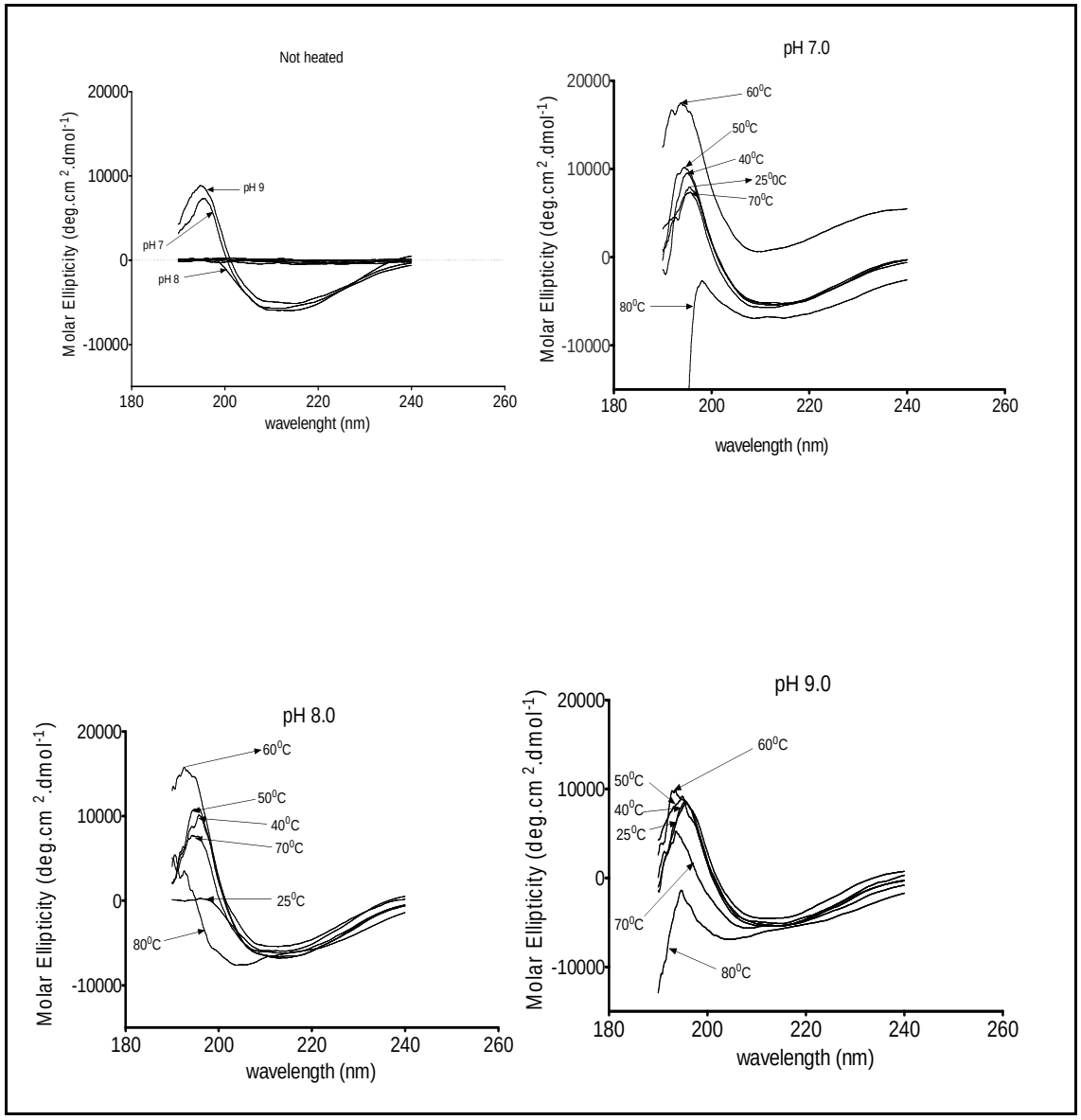


Fig. 4.5: Effect of heat and pH on the near-ultraviolet circular dichorism spectra of kidney bean vicilin

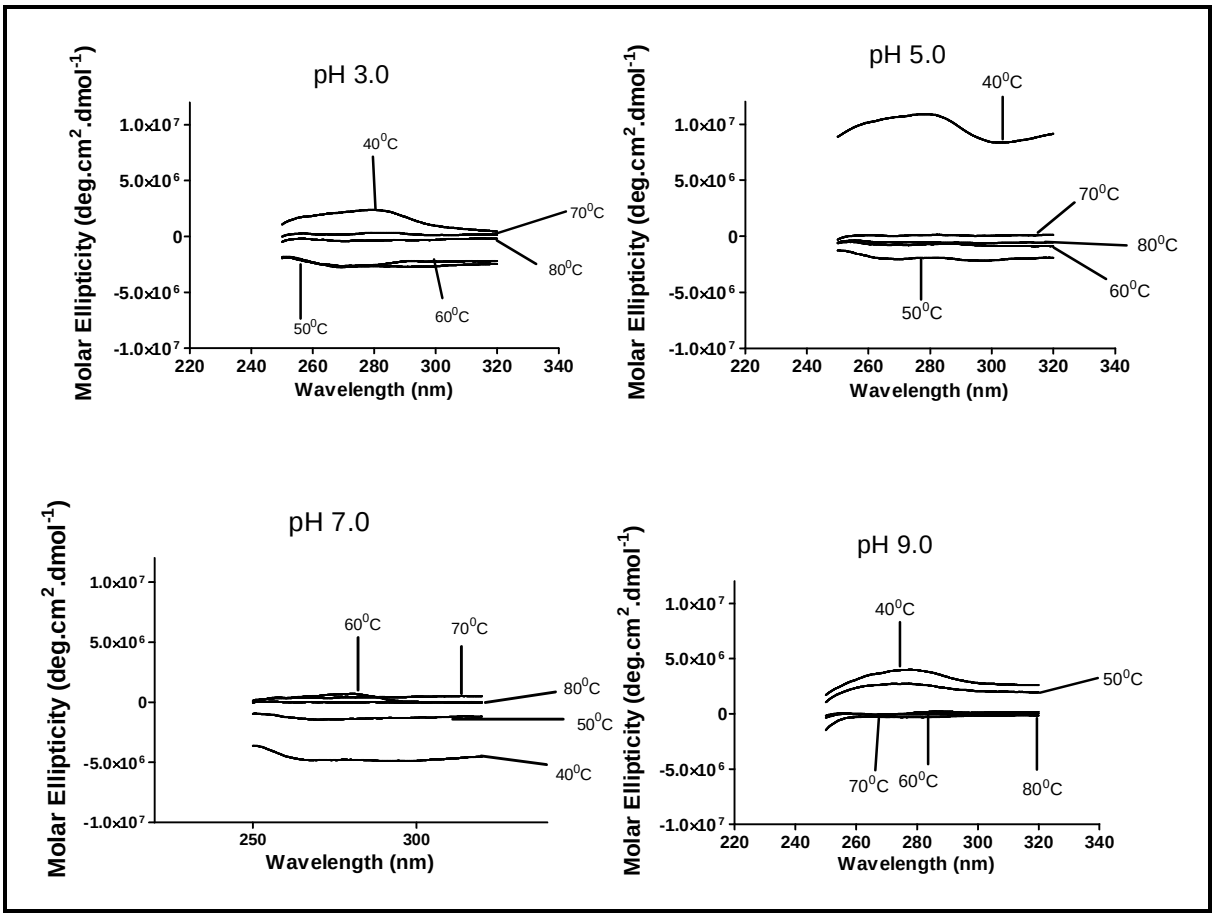
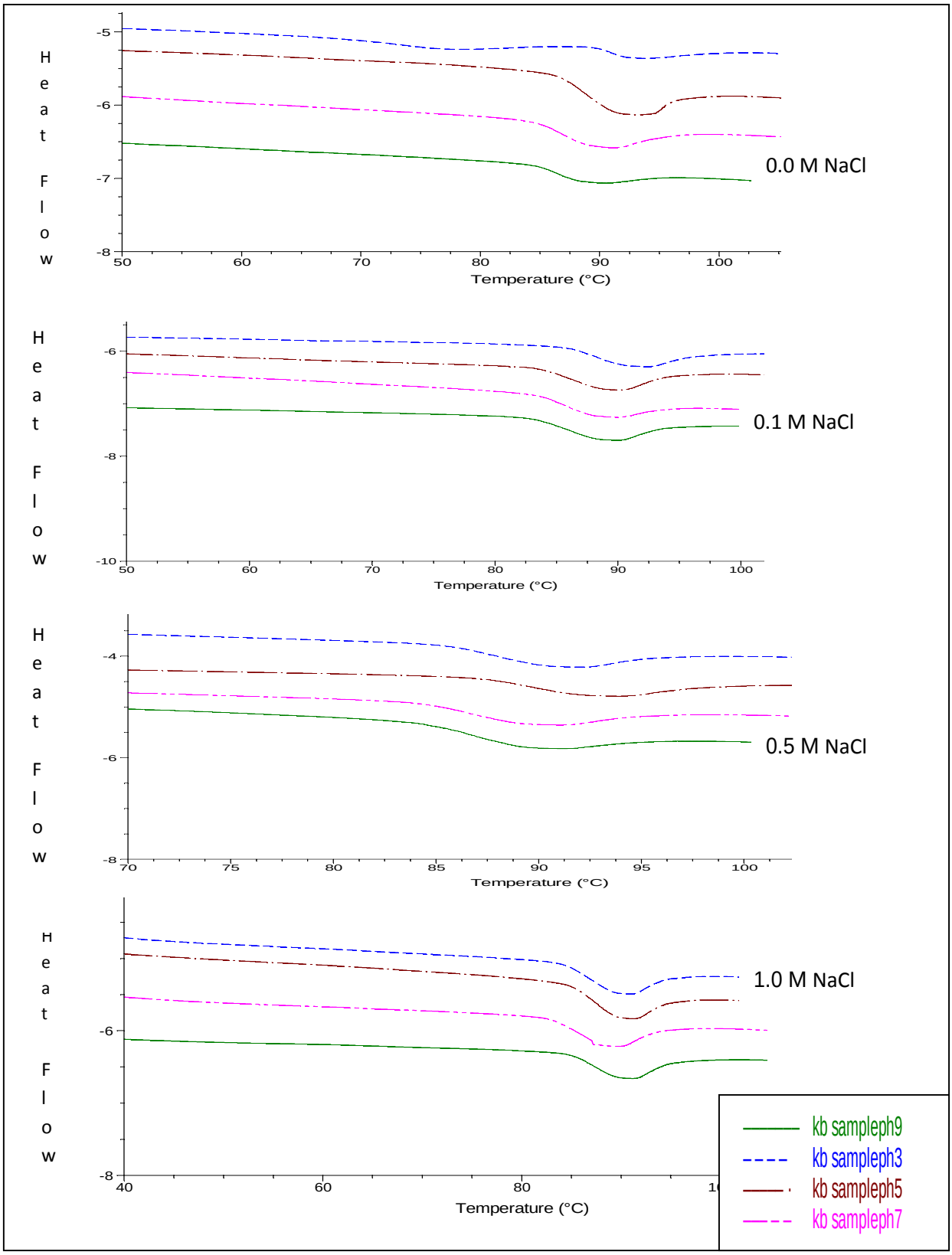


Fig.4.6: Differential scanning calorimetry-thermograms of vicilin (10%, w/v) dispersions as a function of pH and NaCl



References

Acharya, P., Segall, M.L., Zaiou, M., Morrow, J., Weisgraber, K.H., Phillips, M.C., Lund-Katz, S. and Snow, J. 2002. Comparison of the stabilities and unfolding pathways of human apolipoprotein E isoforms by differential scanning calorimetry and circular dichroism. Biochim Biophys Acta *1584*, 9-19.

Adebowale, K.O. and Lawal, O.S. 2003. Foaming, gelation and electrophoretic characteristics of mucuna bean (Mucuna pruriens) protein concentrates. Food Chem. *83*, 237-246.

Aluko, R.E. and Mcintosh, T. 2001. Polypeptide profile and functional properties of defatted meals and protein isolates of canola seeds. J. Sci. Food Agric. *81*, 391-396.

Aluko, R.E., Mofolasayo, O.A. and Watts, B.M. 2009. Emulsifying and foaming properties of commercial yellow pea (Pisum sativum L.) seed flours. J. Agric. Food Chem. *57*, 9793-9800.

Aluko, R.E. and Yada, R.Y. 1995. Structure-function relationships of cowpea (Vigna unguiculata) globulin isolate: Influence of pH and NaCl on physicochemical and functional properties. Food Chem. *53*, 259-265.

Cabra, V., Arreguín, R. and Farres, A. 2008. Emulsifying properties of proteins. Bol. Soc. Quím. Méx. *2*, 80-89.

Carbonaro, M. 2006. 7S globulins from Phaseolus vulgaris L.: Impact of structural aspects on the nutritional quality. Bio. biotechnol. and biochem *70*, 2620-2626.

Chau, C.F. and Cheung, P.C.K. 1998. Functional properties of flours prepared from three Chinese indigenous legume seeds. Food Chem. *61*, 429-433.

Fukuda, T., Maruyama, N., Kanazawa, A., Abe, J., Shimamoto, Y., Hiemori, M., Tsuji, H., Tanisaka, T. and Utsumi, S. 2005. Molecular analysis and physicochemical properties of electrophoretic variants of wild soybean Glycine soja storage proteins. J. Agric. Food Chem. *53*, 3658-3665.

Gauthier, S.F., Paquin, P., Pouliot, Y. and Turgeon, S. 1993. Surface activity and related functional properties of peptides obtained from whey proteins. J. Dairy Sci. *76*, 321-328.

German, B., Damodaran, S. and Kinsella, J.E. 1982. Thermal dissociation and association behavior of soy proteins. J. Agric. Food Chem. *30*, 807-811.

Gill, P., Moghadam, T.T. and Ranjbar, B. (2010). Differential scanning calorimetry techniques: Applications in biology and nanoscience. *21*, 167-193.

Greenfield, N.J. 2007. Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions. Nat. Protocols. *1*, 2527-2535.

Greenfield, N. J. 2004. Circular Dichroism Analysis for protein-protein interactions. In *Protein-Protein Interactions* (Anonymous) pp. 55-77, .

Halling, P.J. 1981. Protein-stabilized foams and emulsions. Crit. Rev. Food Sci. Nutr. *15*, 155-203.

Hayakawa, S. And Nakai, S. 1985. Relationships of hydrophobicity a net charge to the solubility of milk and soy proteins. J. Food Sci. *50*, 486-491.

Kato, A. and Nakai, S. 1980. Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. Biochim. Biophys. Acta. *624*, 13-20.

Kelly, S. M. and Price, N. C. 2006. Overview of Protein Folding. In *Current Protocols in Protein* ***Science*** (G. Taylor, ed.) pp. 20.10.1-20.10.18, John Wiley & Sons, Inc, .

Kelly, S.M., Jess, T.J. and Price, N.C. 2005. How to study proteins by circular dichroism. Biochimica et Biophysica Acta, *1751*, 119-139.

Lawal, O.S., Adebowale, K.O., Ogunsanwo, B.M., Sosanwo, O.A. and Bankole, S.A. 2005. On the functional properties of globulin and albumin protein fractions and flours of African locust bean (*Parkia biglobossa*). Food Chem. *92*, 681-691.

Leon, A., Rosell, C.M. and De Barber, C.B. 2003. A differential scanning calorimetry study of wheat proteins. Eur Food Res Technol *, 217*, 13-16.

Ma, C.Y., Khanzada, G. and Harwalkar, V.R. 1988. Thermal gelation of oat globulin. J. Agric. Food Chem. *36*, 275-280.

Makri, E.A. and Doxastakis, G.I. 2006. Emulsifying and foaming properties of Phaseolus vulgaris and coccineus proteins. Food Chem. *98*, 558-568.

Markwell, M.A.K., Haas, S.M., Bieber, L.L. and Tolbert, N.E. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. *87*, 206-210.

Martin, A.H., Grolle, K., Bos, M.A., Stuart, M.A.C. and Van Vliet, T. 2002. Network Forming Properties of Various Proteins Adsorbed at the Air/Water Interface in Relation to Foam Stability. J. Colloid Interface Sci. *254*, 175-183.

Martin, S. R. and Schilstra, M. J. 2008. Circular Dichroism and Its Application to the Study of Biomolecules. In *Methods in Cell Biology* (Anonymous) pp. 263-293, Academic Press, .

Meng, G. And Ma, C. 2002. Characterization of globulin from Phaseolus angularis (red bean). Int. J. Food Sci. Technol. *37*, 687-695.

Prak, K., Nakatani, K., Katsube-Tanaka, T., Adachi, M., Maruyama, N. and Utsumi, S. 2005. Structure-function relationships of soybean proglycinins at subunit levels. J. Agric. Food Chem. *53*, 3650-3657.

Ragab, D.M., Babiker, E.E. and Eltinay, A.H. 2004. Fractionation, solubility and functional properties of cowpea (*Vigna unguiculata*) proteins as affected by pH and/or salt concentration. Food Chem. *84*, 207-212.

Sai-Ut, S., Ketnawa, P. and Rawdkuen, S. 2009. Biochemical and functional properties of proteins from red kidney, navy and adzuki beans. As. J. Food Ag-Ind. *2*, 493-504.

Salvador, P., Saguer, E., Parés, D., Carretero, C. and Toldrà, M. 2010. Foaming and emulsifying properties of porcine red cell protein concentrate. Food Sci. Technol. Int. *16*, 289-296.

Sathe, S.K. 2002. Dry bean protein functionality. Crit. Rev. Biotechnol. *22*, 175-223.

Siddiq, M., Ravi, R., Harte, J.B. and Dolan, K.D. 2010. Physical and functional characteristics of selected dry bean (Phaseolus vulgaris L.) flours. *43*, 232-237.

Sundd, M., Kundu, S., Dubey, V.K. and Jagannadham, M.V. 2004. Unfolding of ervatamin C in the presence of organic solvents: Sequential transitions of the protein in the O-state. *37*, 586-596.

Tang, C. and Ma, C. 2009. Heat-induced modifications in the functional and structural properties of vicilin-rich protein isolate from kidney (Phaseolus vulgaris L.) bean. Food Chem. *115*, 859-866.

Tang, C., Sun, X. and Foegeding, E.A. 2011. Modulation of physicochemical and conformational properties of kidney bean vicilin (Phaseolin) by glycation with glucose: implications for structure-function relationships of legume vicilins. J. Agric. Food Chem. *59*, 10114-10123.

Townsend, A. and Nakai, S. 1983. Relationships between hydrophobicity and foaming characteristics of food proteins. J. Food Sci. *48*, 588-594.

Wilde, P., Mackie, A., Husband, F., Gunning, P. and Morris, V. 2004. Proteins and emulsifiers at liquid interfaces. Adv. Colloid Interface Sci. *108–109*, 63-71.

Yin, S., Tang, C., Wen, Q. and Yang, X. 2010. Functional and conformational properties of phaseolin (*Phaseolus vulgaris* L.) and kidney bean protein isolate: A comparative study. J. Sci. Food Agric. *90*, 599-607.

Zhang, M., Gao, J. and Yang, H. 2009a. Functional properties of 7S globulin extracted from cowpea vicilins. Cereal Chem. *86*, 261-266.

Zhang, T., Jiang, B., Mu, W. And Wang, Z. 2009b. Emulsifying properties of chickpea protein isolates: Influence of pH and NaCl. Food Hydrocoll. *23*, 146-152.

CHAPTER 5

INHIBITORY PROPERTIES OF KIDNEY BEAN PROTEIN HYDROLYSATE AND ITS MEMBRANE FRACTIONS AGAINST RENIN, ANGIOTENSIN CONVERTING ENZYME, AND FREE RADICALS

S. Mundi and R.E. Aluko*

The Department of Human Nutritional Sciences and the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

*Corresponding author: Rotimi E. Aluko, Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, MB R3T 2N2, Canada; E-mail: alukor@cc.umanitoba.ca, Tel: +1(204) 474-9555, Fax: +1(204) 474-7593

To be submitted for publication

5.0. **Abstract**

Kidney bean hydrolysate (KBH) was obtained by alcalase hydrolysis of the globulin proteins followed by membrane ultrafiltration to produce peptide fractions that differ in molecular sizes (<1, 1-3, 3-5, and 5-10 kDa). Evaluation of potential antihypertensive properties of the peptides showed that the <1 and 5-10 kDa fractions exhibited significantly highest ($p<0.05$) renin inhibition. In contrast, the KBH and peptide fractions showed similar and non-significant ($p<0.05$) inhibitory activities against angiotensin converting enzyme. The antioxidant power of the hydrolysates was evaluated through scavenging of the DPPH free radical, inhibition of peroxidation of linoleic acid, hydroxyl radical scavenging, ferric reducing power and metal chelation. The <1 and 5-10 kDa peptide fractions showed significantly ($p<0.05$) higher ability to scavenge DPPH free radical, inhibit peroxidation of linoleic acid and reduce Fe^{3+} to Fe^{2+}. Generally the fractions with <1 and 5-10 kDa peptides showed better potential as antihypertensive and antioxidant peptides, probably due to their slightly higher contents of hydrophobic amino acids. It was concluded that kidney bean protein hydrolysate and peptide fractions could potentially serve as useful ingredients to formulate functional foods and nutraceuticals against hypertension and oxidative stress.

Key words: Kidney bean, hydrolysate, alcalase, hydrolysis, globulin, ultrafiltration, peptides

5.1. Introduction

Several food proteins and peptides have been shown to display specific biological activities in addition to their proven nutritional value (Hartmann & Meisel, 2007; Mine, Li - Chan, & Jiang, 2010; Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008; Xiong, 2010). A growing body of scientific evidence is continuously revealing the positive impact of bioactive peptides and proteins on body function and human health by alleviating conditions such as coronary (ischemic) heart disease, stroke, hypertension, cancer, obesity, diabetes, and osteoporosis (see review articles by Mine et al., 2010; Udenigwe and Aluko, 2011). Specifically, research studies have shown some evidence for the efficiency of plant protein-derived peptides in improving hypertension or contributing to the overall antioxidant capacity of cells (Li et al. 2011; Medina-Godoy et al., 2012; Udenigwe, Lin, Hou, & Aluko, 2009; Udenigwe et al. 2011). It has been reported that a large range of antihypertensive and antioxidant peptides and peptide mixtures (hydrolysates) have been produced from various food products such as beans, soy, corn, potato, peanut, milk, whey, egg, and meat proteins (Xiong, 2010). These peptides are inactive within the sequence of their parent proteins but can be released by chemical, enzymatic and microbial methods (Peñta-Ramos & Xiong, 2002; Xiong, 2010). By far, the most effective and dependable method to produce peptides with the intended functionalities is by enzymatic digestion (Xiong, 2010). Bioactive peptides are either produced *in vivo* by the action of gastrointestinal enzymes or obtained *in vitro* using specific enzymes, or during the preparation of certain foods. However, the source of proteins, the protein substrate pretreatment, the type of enzymes used, and the hydrolysis conditions applied, all affect the efficacy of protein hydrolysates and peptides produced

from them (Xiong, 2010). It is also known that the nature of residues in a peptide influences its activity (Xiong, 2010). These peptides have the advantage of being naturally derived from food protein sources normally consumed as part of the daily diet, and they are considered to be milder and safer without the side effects associated with drugs. Peptides with antioxidant and ACE-inhibitory activities are usually rich in hydrophobic amino acids and many of them contain proline, lysine or arginine as C-terminal residues (Matsui & Tanaka, 2010; Xiong, 2010).

High blood pressure confers a high risk of complications, as it is one of the major risk factors for cardiovascular diseases including coronary heart disease, peripheral artery disease and stroke (Kodera & Nio, 2006; Matsui & Tanaka, 2010; Nakahara et al., 2010). Clinical evidence has shown that peptides released by the action of enzymes could be involved in the inhibition of the renin-angiotensin-aldosterone system, which is one key pathway for combating hypertension. Angiotensin converting enzyme (ACE; dipeptidylcarboxypeptidase I; EC 3.4.15.1) is well-known for its physiological role in the renin-angiotensin system (Hooper, 1991; Kitts & Weiler, 2003; Nigel, 1991). ACE acts by removing a dipeptide from the C-terminal of angiotensin I to generate angiotensin II, the latter being a very potent vasoconstrictor which also stimulates the release of aldosterone from the adrenal cortex leading to sodium ion retention (Matsui & Tanaka, 2010). In addition, ACE is also responsible for inactivating the vasodilator bradykinin (Nigel, 1991). For this dual role in the maintenance of blood pressure and fluid and electrolyte homeostasis, inhibition of ACE has been successfully used for the treatment of hypertension and congestive heart failure (Nigel, 1991). Synthetic ACE inhibitors such as captopril, enalapril, lisinopril and ramipril have been widely used for the effective

clinical treatment of hypertension and heart failure in humans, however they are also associated with disadvantages, such as easy digestion by protease in the body, coughing, allergies, taste disturbances, and skin rashes, impaired renal function, and especially excessively low blood pressure, i.e. hypotension (Kitts & Weiler, 2003; Matsui & Matsumoto, 2006). For this reason, identification of possible natural sources of ACE inhibitors that have a strong antihypertensive activity and resistance to digestion by various proteases and with minimal negative side effect will be of great interest to formulators of functional foods. Although the effectiveness of the ACE-inhibitory activity may not be as high as those of synthetic drugs, many natural ACE-inhibitory peptides isolated from different food proteins could be applied in the prevention of hypertension and in the initial treatment of mildly hypertensive individuals (Li, Jiang, Zhang, Mu, & Liu, 2008). Amongst the ACE peptide inhibitors, the majority are di- or tripeptides, which are resistant to the digestive tract endopeptidases and can be easily absorbed into the blood (Matsui & Matsumoto, 2006; Matsui & Tanaka, 2010).

Peptides have also been shown to be capable of inhibiting the uncontrolled oxidation of the biomacromolecules usually caused by reactive oxygen species (ROS). They are known to act against an oxidative sequence by terminating chain reactions and removing free radical intermediates; therefore, they are able to reduce intensity of diseases like cancer, heart disease etc. Bioactive peptides block the oxidation process by neutralizing free radicals such as superoxide anion radical ($O2^{-}$) and hydroxyl radical ($^{\cdot}OH$) which are products of regular metabolism (Nazeer & Srividhya, 2011). Several *in vitro* studies have produced evidence that peptides generated from certain food proteins by enzymatic hydrolysis, including quinoa seed proteins (Aluko & Monu, 2003;

Amarowicz & Shahidi, 1997), capelin protein (Amarowicz & Shahidi, 1997), canola (Cumby, Zhong, Naczk & Shahidi, 2008) and egg-yolk protein (Sakanaka, Tachibana, Ishihara & Juneja, 2004) possess strong antioxidant activities. In particular, published studies have revealed strong evidence for the antioxidant activity for legumes such as chickpeas (Aluko, 2008; Li et al., 2008; Li, Liu, Zhang, Jiang, & Mu, 2008; Medina-Godoy et al., 2012; Megías et al., 2007; Yust, Millán-Linares, Alcaide-Hidalgo, Millán, & Pedroche, 2012; Zhang, Li, Miao, & Jiang, 2011) and soybean (Gibbs, Zougman, Masse, & Mulligan, 2004), but not much is known about the antioxidant and antihypertensive activity of kidney bean protein hydrolysate and the effect of peptides size on potency.

Kidney bean (*Phaseolus vulgaris*) is a pulse crop that contain high amount of proteins (20–30%) on a dry weight basis (Yin, Tang, Yang, Wen, & Qi, 2011). This puts them among some of the richest food sources of proteins, making them a good candidate to explore for the production of bioactive peptides. The goal of this study was to obtain protein hydrolysates from kidney bean globulin, undertake membrane separation of the hydrolysate by the use of specific molecular weight cut-offs and to analyze their potential for the inhibition of enzymes associated with the renin-angiotensin-aldosterone system. In addition, different measurements, including the ability to inhibit the autoxidation of linoleic acid, the scavenging effect on the DPPH free radical, and the reducing power were used to evaluate the antioxidant activities of the peptides produced.

5.2. Materials and Method

5.2.1. Materials

Red kidney bean seeds were obtained from a local store in Winnipeg while alcalase, N-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG), glutathione (GSH), DPPH (2, 2-Diphenyl-1-picrylhydrazyl), 1-anilino-8-naphthalene sulfonate (ANS) and ACE from rabbit lung (E.C.3.4.15.1) were purchased from Sigma-Aldrich (St. Louis, MO). Human recombinant Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Other analytical grade reagents and ultrafiltration membranes were obtained from Fisher Scientific (Oakville, ON, Canada).

5.2.2. Extraction and isolation of globulin proteins

Red kidney bean seeds were ground into flour using centrifugal mill (Retsch ZM200). Globulin proteins were extracted from the flour according to the previously described ammonium sulfate precipitation method (Aluko, 2004). The major globulin fraction in kidney beans was obtained by adjusting an aqueous extract (obtained using 0.1 M phosphate buffer, pH 7.0 containing 0.4 M NaCl) of the flour to 40% ammonium sulfate saturation, in order to precipitate smaller proteins and enzymes. After centrifugation (7000x*g,* 1 h, 4^{o}C), the supernatant was then brought to 80% ammonium sulfate saturation to precipitate the globulins. The precipitating salt (ammonium sulfate) was then removed from the isolated globulins by dialyzing sample against water. The dialysis bag content was centrifuged (7000x*g,* 1 h, 4^{o}C) and the resultant precipitate was freeze-dried as the globulin isolate.

5.2.3. Preparation and fractionation of kidney bean globulin protein hydrolysates

Proteolysis of the isolated kidney bean globulin isolate was conducted with alcalase. The globulin protein isolate (5%, w/v, protein weight basis) was suspended in deionized water

in a reaction vessel equipped with a stirrer, heated to 37^{o}C and adjusted to pH 9.0 prior to the addition of alcalase (4% w/w, based on the protein content of the protein isolate). The digestion was performed at the above stated conditions for 4 h with the pH of the reaction mixture maintained constant by addition of 2 M NaOH. At the end of the proteolysis period, the mixtures were heated in boiling water for 10 min to inactivate alcalase and by adjusting to pH 4.0 with 2 M HCl to precipitate the undigested proteins; thereafter, the hydrolysates were centrifuged (30 min at 7,000×*g*). The supernatant containing target peptides was collected as the kidney bean protein hydrolysate (KBH) and passed through a 1 kDa membrane with the retentate passed through a 3 kDa ultrafiltration membrane. The retentate from 3 kDa membrane was passed through a 5 kDa whose retentate was then passed through a 10 kDa membrane. Permeates collected from each membrane were designated as <1, 1-3, 3-5 and 5-10 kDa peptide fractions, respectively. The protein hydrolysate and the various membrane fractions were freeze-dried and protein contents were determined by the modified Lowry's method using bovine serum albumin as a standard (Markwell, Haas, Bieber, & Tolbert, 1978). Also, the percentage gross yield of hydrolysate was calculated as weight of dried sample divided by weight of globulin isolate used for hydrolysis. Yields of the ultrafiltration fractions were calculated as weight of dried fraction divided by weight of KBH that was used for filtration.

5.2.4. Amino acid analysis

HPLC system was used for the analysis of the amino acid profiles after samples were hydrolyzed with 6 M HCl according to the method of (Bidlingmeyer, Cohen, & Tarvin, 1984). The cysteine and methionine contents were determined after performic acid

oxidation (Gehrke, Wall Sr., & Absheer, 1985) while tryptophan content was determined after alkaline hydrolysis (Landry & Delhaye, 1992).

5.2.5. Surface hydrophobicity (S_o) determination

Surface hydrophobicity of the KBH and the ultrafiltration fractions was determined using a hydrophobic fluorescence probe, 1-anino-8-naphthalene sulfonate (ANS) method as described by (Hayakawa & Nakai, 1985) with some modifications. Samples were serially diluted to 0.0025-0.015% (w/v) in 0.01 M phosphate buffer (pH 7.0). Twenty µl of ANS (8.0 mM in 0.1 M phosphate buffer, pH 7.0) were added to 2 ml protein solution. Fluorescence intensity of ANS-protein conjugates were measured with JASCO FP-6300 spectrofluorimeter (JASCO, Tokyo, Japan) at the excitation and emission wavelengths of 390 and 470 nm, respectively.

5.2.6. Determination renin inhibitory activity

The method of Li & Aluko (2010) was used to perform the renin inhibition assay using the Renin Inhibitor Screening Assay Kit (Cayman Chemicals, Ann Arbor, MI). The blank was prepared by adding 20 µl of substrate, 160 µl of assay buffer, and 10 µl of Milli-Q water to the background wells. Thereafter, an aliquot of 20 µl of substrate, 150 µl of assay buffer, and 10 µl of Milli-Q water were added to the control wells while 20 µl of substrate, 150 µl of assay buffer, and 10 µl of KBH and peptide fractions samples were added to the inhibitor wells. The reaction was initiated by adding 10 µl of renin to the control and sample wells. The microplate was shaken for 10 s to mix, incubated at 37°C for 15 min, and then fluorescence intensity (FI) was recorded using an excitation wavelength of 340 nm and emission wavelength of 490 nm.

The percentage inhibition was calculated as:

$$\% \text{ Renin inhibition} = \frac{\text{FI (control) - FI (sample)}}{\text{FI (control)}} \times 100$$

5.2.7. Determination of ACE- inhibitory activity

The ACE-inhibitory activity was assayed as described by Udenigwe et al (2009) using FAPGG as substrate. ACE catalyzes the hydrolysis of FAPGG, forming furylacryloylphenylalanine (FAP). The decrease of the absorbance at 340 nm is proportional to the activity of the ACE. Briefly, 1 ml of 0.5 mM FAPGG (dissolved in 50 mM Tris–HCl buffer containing 0.3 mM NaCl, pH 7.5) was mixed with 20 µl of ACE (1 U/ml; final activity of 20 mU) and 200 µl of KBH and peptide fractions in 50 mM Tris–HCl buffer. The decrease in absorbance at 340 nm, due to cleavage of the Phe-Gly peptide bond of FAPGG, was recorded for 2 min at room temperature. For the control experiment, Tris–HCl buffer was used instead of peptide fraction solutions. All experiments were performed in triplicate. The percentage inhibition of ACE was calculated as:

$$\%\text{ACE inhibition} = \frac{\text{Abs of control-Abs of sample}}{\text{Abs of control}} \times 100$$

5.2.8. DPPH radical scavenging assay

Reduction of DPPH by an antioxidant usually results in a loss of absorbance at 517 nm. The extent of discoloration of the solution indicates the scavenging efficiency of the added compound. Determination of antioxidant activity of KBH and the peptide fractions was adapted from the method described by Hou et al (2001) using a 96-well microplate. The KBH and the peptide fractions were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 containing 1% (w/v) Triton X-100. A solution of DPPH was prepared in methanol to

a final concentration of 100 μM. Samples or GSH (100 μL; final assay concentration of 1 mg/mL) was added to 100 μL of DPPH in a 96-well microplate. A control well contained only DPPH and the sodium phosphate buffer. The plate was then covered and incubated in the dark at room temperature for 30 min; absorbance of the sample (As) and control (Ac) at 517 nm was measured in a spectrophotometer. The scavenging activities of KBH and the peptide fractions were compared to that of GSH. The percent scavenging activity of GSH and the samples was calculated using the following equation:

DPPH Radical Scavenging Activity (%) = (Ac-As/Ac) × 100

5.2.9. Determination of Fe^{2+} chelating activity

The iron chelating activity of KBH and peptide fractions was measured following the ferrozine method as described by Ajibola et al. (2011). KBH, peptide fraction or GSH solutions (final concentration of 1 mg/mL) was mixed with 0.05 mL of 2 mM $FeCl_2$ and 1.85 mL distilled water in a reaction tube. Thereafter, 0.1 mL of 5 mM Ferrozine solution was added and mixed thoroughly. The mixture was allowed to stand at room temperature for 10 min followed by removal of 200 μL aliquot of the reaction mixture and added to a clear bottom 96-well plate. The control experiment contained all the reaction mixtures except that distilled water was used to replace the sample. Absorbance of sample (As) and control (Ac) was measured using a spectrophotometer at 562 nm and the metal chelating activity of the sample was compared to that of GSH. The percentage chelating effect (%) was calculated using the following equation:

Fe^{2+} chelating activity (%) = (Ac-As/Ac) × 100

5.2.10. Ferric reducing power assay

The ability of the hydrolysate to reduce iron (III) was determined according to the method of Yildirim, Mavi, and Kara (2001) with some modifications. Different concentrations of KBH, peptide fractions or GSH (1, 5 and 10 mg/mL) in 250 µL of distilled water were mixed with phosphate buffer (250 µL of 0.2 mM, pH 6.6) and 250 µL of 1% potassium ferricyanide solution dissolved in distilled water. The mixture was incubated at 50^{o}C for 30 min, followed by addition of 250 µL of 10% (w/v) trichloroacetic acid. The mixture was then centrifuged at 1000x*g* for 10 min. Finally, 250 µL of the supernatant solution was mixed with 50 µL of distilled water and 50 µL of 0.1% (w/v) ferric chloride solution followed by addition of distilled water (200 µL). After 10 min reaction the absorbance of the resulting solution was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

5.2.11. Inhibition of hydroxyl radical formation.

The hydroxyl radical scavenging activity was measured according to the protocol previously described (Ajibola et al., 2011). KBH, peptide fractions, GSH and 1, 10-phenanthroline (3 mM) were each separately dissolved in 0.1 M sodium phosphate buffer (pH 7.4) while FeSO4 (3 mM) and 0.01% hydrogen peroxide were each separately dissolved in distilled water. Fifty µl of 1, 10-phenanthroline and 50 µL of FeSO4, were added consecutively to 50 µL aliquot (equivalent to a final assay concentration of 1 mg/mL) of KBH, peptide fractions, GSH, or buffer (control) in a clear, flat bottom 96-well microplate. To initiate reaction in the wells, 50 µL of hydrogen peroxide (H_2O_2) solution was added to the mixture, which was then covered and incubated at 37^{o}C for 1 h with shaking. Thereafter, the absorbance of the mixtures was measured at 536 nm every 10 min for a period of 1 h. The absorbance was also determined for a blank (without

peptides or H_2O_2) and a control (without peptides). The $OH^{\cdot}$ scavenging activity was calculated as described by Ajibola et al. (2011).

5.2.12. Inhibition of Linoleic Acid Oxidation

Linoleic acid oxidation was measured using a previously described method (Ajibola et al. 2011). KBH, peptide fractions or GSH were dissolved in 1.5 mL of 0.1 M phosphate buffer, pH7.0 at a final concentration of 1 mg/mL. Each mixture was added to 1 mL of 50 mM ethanolic linoleic acid and stored in a glass test tube kept at 60^{o}C in the dark for 7 days. On a daily basis, 100 µL of the reaction mixture was removed and mixed with 4.7 mL of 75% aqueous ethanol, 0.1 mL of ammonium thiocyanate (30%, w/v) and 0.1 mL of 0.02 M acidified ferrous chloride (dissolved in 1 M HCl). An aliquot (200 µL) of the resulting solution was added to a clear bottom 96-well microplate and the degree of color development was measured using the spectrophotometer at 500 nm after 3 min incubation at room temperature

5.2.13. Statistical Analysis

Data were collected in triplicates and subjected to one way analysis of variance using Statistical Analysis System Software (SAS version 9.2, SAS Institute, Cary, NC). Significant differences were determined by Duncan's multiple range test and accepted at $p<0.05$.

5.3. Result and discussion

5.3.1. Protein hydrolysis

Enzymatic proteolysis of kidney bean globular proteins and subsequent fractionation of the resultant KBH by ultrafiltration resulted in fractions rich in small size (<10 kDa) peptides. The percent gross yield of KBH was 78%, and approximately 30.7, 20.3, 17 and

18% of peptides in the KBH had molecular weights of <1, 1-3, 3-5 and 5-10 kDa, respectively. The final retentate (>10 kDa fraction), which contained large size peptides had a yield of 14%. The protein contents were 87, 96, 89, 78 and 90% for the <1, 1-3, 3-5, 5-10 kDa and the KBH, respectively. The high yield of KBH reflects efficient digestion of the globular proteins by alcalase.

5.3.2. Amino acid analysis

Amino acid analysis of the unhydrolyzed and alcalase-treated globulins from kidney bean seed as well as the peptide fractions collected as permeates from 1, 3, 5 and 10 kDa membrane cut-offs are shown in Table 5.1. The amino acid analysis of the unhydrolyzed globulin meets the FAO's 35% recommendations of essential amino acid (FAO/WHO, 1985). Protease hydrolysis of the globulin proteins affected the amino acid content of the fractions in various ways. Generally, the unhydrolyzed globulin and KBH as well as the peptide fractions all contained low levels of methionine and cysteine, which is typical of legume proteins. Conversely, all samples had high contents of glutamic acid, glutamine, aspartic acid, asparagine, lysine and alanine. Except for proline, cysteine, isoleucine valine and histidine which were relatively higher, most of the amino acid contents of the KBH were slightly lower than the unhydrolyzed globulin. In contrast, Pownall et al. (2010) investigated the amino acid composition of pea peptides fractions separated from the <3 kDa permeate using high performance liquid chromatography (HPLC) and found an increase in hydrophobic (both aliphatic and aromatic) amino acid of the hydrolysate when compared with the isolate. In our study, when compared to the KBH, most peptide fractions showed higher average hydrophobicity. Pownall et al. (2010) and Megías et al (2009) also reported higher contents of certain hydrophobic amino acids in relation to the

original protein hydrolysate. Hydrolysis markedly increased the cysteine content of the KBH and all the peptide fractions even though the other sulphur-containing amino acid, methionine was slightly reduced in all the fractions. The total hydrophobic aliphatic amino acid (valine, isoleucine and leucine) content showed the highest concentration in the <1 kDa fraction. The percentage of valine also increased in the hydrolysate and in the polypeptides with 5-10 kDa size, but was least in the 3-5 kDa fraction. The percentage content of leucine and isoleucine residues as well as hydrophobic aromatic amino acid (phenylanaline and tyrosine) were highest in the <1 kDa fraction, decreasing as the membrane MW cut-off increased from 1 kDa to 10 kDa. Proline, a slightly hydrophobic amino acid increased in the peptide fractions as MW cut-off was increased. Asparagine/aspartic acid and glutamine/glutamic acid contents were also increased in the peptide fractions with exception of the 5-10 kDa fractions.

5.3.3. Surface Hydrophobicity

Proteolysis of proteins may cause changes in protein globular structure as the hydrophobic regions hidden within the native protein are exposed (Medina-Godoy et al., 2012; Wu et al., 1996). After proteolysis, more hydrophobic amino acids are exposed because of the catalytic specificity of alcalase, which cleaves peptides bond formed by these specific amino acid residues (Medina-Godoy et al., 2012; Wu, Aluko, & Muir, 2009). A quantitative structure-activity relationship study of ACE-inhibitory peptides confirmed that peptides composed of amino acids with strong hydrophobicity at their C- and N-terminal have potent ACE–inhibitory activities. Hydrophobicity is also a very important contributing factor to the activity of antioxidative peptides (Liu et al., 2005). This is because, the surface hydrophobic site is partly responsible for formation and

maintenance of the spatial structures as well as protein interactions, including binding to cell membranes, protein–protein recognition, and formation of complexes with biologically active compounds (Voronov, Skirgello, Troshina, Orlova, & Kost, 2002). Since the hydrophobic interactions are the driving forces for manifestation of the physiological functions of peptides, information on the hydrophobic character of bioactive peptides could contribute to further understanding of their mechanism of action. To determine hydrophobicity of the peptides, a hydrophobic fluorescent dye (ANS) was used as a probe. As shown in Figure 5.1, the affinity of ANS for hydrophobic patches increased from the <1 kDa fraction to the 5-10 kDa. This is probably because there are more oligopeptides available as the size of the membranes increased with larger surface containing the exposed hydrophobic residues. Wu, Hettiarachchy, & Qi (1998) also observed a decrease in the surface hydrophobicity of smaller soy peptides prepared by longer time papain hydrolysis compared to the larger peptides produced by shorter time hydrolysis. The authors attributed the observed difference to fewer hydrophobic binding sites on the smaller peptides compared to the larger peptides. The same authors (Wu et al., 1998) also observed lower surface hydrophobicity for the ultrafiltrates than the hydrolysates. On a similar note Molina Ortiz & Wagner, (2002) also observed that, smaller chain peptide species had less surface hydrophobicity. However, our results differ from those reported by Wang and co-workers (2007) for papain hydrolysates of wheat gluten who showed that the permeate with a molecular weight cut-off of 5 kDa had higher surface hydrophobicity than the hydrolysate. The trend for the surface hydrophobicity is not consistent with the hydrophobic amino acid residue content, which suggests that arrangement of the amino acids differ between the peptide chains.

5.3.4. Renin Inhibitory Activity

Ultrafiltration separation of the KBH led to increased renin-inhibitory activities of the peptide fractions as shown in Fig. 5.2. Renin inhibitory activity is related mostly to the amino acid contents of the inhibitory peptides (Udenigwe et al., 2011). As indicated by the amino acid analysis (Table 5.1), peptides fraction with molecular masses less than 1 kDa and 5-10 kDa have higher contents of hydrophobic (valine, isoleucine and leucine) and aromatic amino acids (phenylanaline and tryptophan). Therefore, the higher renin-inhibitory activities of the <1 kDa and 5-10 kDa peptide fractions may be attributed to the increased levels of hydrophobic and aromatic amino acids when compared to the 1-3 and 3-5 kDa peptide fractions. Yuan et al. (2006) reported similar inhibitory capacity to the values reported for <1 kDa and 5-10 kDa peptide in our current study, and the authors associated the inhibitory potency of the samples tested to the hydrophobicity of the amino acid, especially at the N-terminal. Similar to the report by Udenigwe et al. (2009), it is speculated that the nature and position of the amino acid rather than size of peptides play a major role in the current study for their inhibitory activity. In a quantitative structure–activity relationship modeling of renin-inhibiting dipeptides, Udenigwe et al. (2011) suggested the need for the presence of a highly hydrophobic, low molecular size N-terminal amino acid (e.g. isoleucine) in renin-inhibiting dipeptides.

5.3.5. ACE-Inhibitory Activity

Stable blood pressure is usually maintained by the hypertensive peptide, angiotensin II, and the hypotensive peptide, bradykinin (Jang et al., 2011). ACE–inhibitory peptides that have shown very important role in regulating blood pressure through the direct action of angiotensin II on blood vessels range from dipeptides to oligopeptides (Jang et al., 2011).

In the current study, the ability of KBH and peptide fractions to inhibit the *in vitro* activity of ACE is shown in Fig5.3. All peptide fractions and the KBH showed high percentage (above 77%) of ACE-inhibitory activity. The highest ACE inhibition of 80% was shown by KBH while <1 and 3-5 kDa fractions showed the minimum inhibition at 77%: however, there was no significant ($p>0.05$) difference between the ACE-inhibitory activities of all the samples. The peptide fractions and KBH showed higher ACE-inhibitory activity than mung bean hydrolysate prepared with neutrase (Li, Li, He, & Qian, 2011). The differences in these findings may be explained by differences in the enzyme type, the concentration of the sample used, as well as the enzyme-to-substrate ratio, all of which could affect the ACE-inhibitory activity. Compared to the current study, Valdez-Ortiz (2012) reported higher inhibitory activity (90-99%) for alcalase and thermolysin treated Azufrado (sulphur yellow) beans, which was higher than the pancreatin treated bean samples (44-58%). In contrast to the ACE-inhibitory activity reported for hard-to-cook black bean hydrolysates fractions (Ruiz-Ruiz, Dávila-Ortíz, Chel-Guerrero, & Betancur-Ancona, 2012) which was significantly dependent on peptide fraction molecularweight, the inhibitory activity in the present study had no particular trend. When a fraction contains high contents of the positively charged amino acids with a lesser content of the hydrophobic amino acids (Table 5.1), there could be inconsistent trend in observed ACE-inhibitory activities (Wu et al., 2006). Owing to the fact that the substrate for ACE possess negatively charged terminal carboxyl groups (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980), fractions with high proportion of positively charged amino acids may affect the binding capacity of substrate to ACE through interference with the ionic bond. In addition, depending on the hydrophobic amino acid content of the

fractions, hydrophobicity of peptides also plays an important role in determining the potency of ACE-inhibitory peptides (Cheung, Cheung, Tan, & Li-Chan, 2012).

5.3.6. Antioxidant activity of the peptides

Published studies have related antioxidant activity of peptides to the amino acids present in the sequence (Medina-Godoy et al., 2012). For instance, aromatic and sulfur-containing amino acids have the capacity to donate protons to free radicals; this capacity is also influenced by the peptide bond and its conformation (Medina-Godoy et al., 2012; Wu et al., 2009). An additional factor is the location of the amino acids in the peptide sequence, for example, valine and leucine have more antioxidant activity when they are found in the N-terminal position (Medina-Godoy et al., 2012; Wu et al., 2009). Also, peptides that have tryptophan and tyrosine in the C-terminal position have good antioxidant activity; these types of peptide can be released by alcalase, because it hydrolyses the peptide bonds formed by hydrophobic amino acid residues (Medina-Godoy et al., 2012; Wu et al., 2009).

5.3.6.1. DPPH radical scavenging assay

The free radical scavenging activity of KBH and peptide fractions was tested by measuring their ability to quench DPPH radical. The DPPH radical is reduced by conversion to a colourless product in the presence of antioxidants that possess hydrogen-donating or chain-breaking properties (Corrêa et al., 2011). This assay provides stoichiometric information with respect to the number of electrons taken up by the tested compounds in the presence of the stable free radical. DPPH radical-scavenging activities of different fractions and the hydrolysate are shown in Fig. 5.4. Peptide fractions, but not hydrolysates possessed moderate to mild DPPH scavenging activities. The results varied

depending on the peptide size, with the <1 kDa and 5-10 kDa showing 13 and 14%, which is less than 50% of the 32% scavenging capacity obtained for the positive standard (GSH). The DPPH inhibitory activities of 1-3 and 3-5 kDa fractions were significantly lower ($p<0.05$) than activities of <1 and 5-10 kDa fractions. Unlike the study by Girgih, Udenigwe and Aluko (2011) who reported that the DPPH inhibitory activity of hemp peptides depended on the molecular size, the observed pattern of DPPH-scavenging in the current study showed no relationship with molecular size, because activity significantly decreased ($p<0.05$) from 1 kDa to 1-3 kDa but was increased only slightly for the 3-5 kDa fraction and significantly for the 5-10 kDa fraction. The KBH which is a mixture of peptides including <1 to the 5-10 kDa sizes did not demonstrate any ability to scavenge free radical, possibly because of dilution effect, i.e. the ratio of inactive peptides was higher than that of active peptides. Thus, the ultrafiltration-mediated separation led to reduced ratio of the inactive peptides in the peptide fractions. In general, the peptide fractions from KBH were able to act as a DPPH radical scavengers probably because of their electron-donating capacity, and as result, form stable radicals or nonreactive conjugates through radical-peptide condensation (Xiong, 2010). The presence of certain amino acid residues, notably histidine, tyrosine, tryptophan, methionine, cysteine, and proline, amongst other aromatic and hydrophobic amino acids, have been reported to be associated with radical quenching activity of peptides (Peñta-Ramos & Xiong, 2002; Xiong, 2010). The higher DPPH inhibitory activity shown in this study for <1 and 5-10 kDa peptide fractions could have been due to higher contents of hydrophobic aliphatic (valine, isoleucine and leucine) and hydrophobic aromatic (phenylanaline and tyrosine) amino acid residues in the two fractions, when compared to

the 1-3 and 3-5 kDa fractions. The DPPH radical scavenging activity of KBH was lower than 48-58% for barley hydrolysate at 0.5 mg/mL (Bamdad, Wu, & Chen, 2011) and 78.3%, 73.6% and 43.1% at 1.5 mg/ml of smooth hound (*Mustelus mustelus*) muscle protein hydrolysates (Bougatef et al., 2009).

5.3.6.2 Fe^{2+} chelating activity

Studies with rodents (Morita, Kimura, & Itokawa, 1994; Takahashi et al., 2001), complemented by post-mortem human brain tissue analyses (Zecca, Youdim, Riederer, Connor, & Crichton, 2004) have shown that aging is accompanied by a rise in the levels of iron and other metals (e.g., copper, zinc) in the biological system. Iron has been found to be involved in several key pathogenic processes in a number of degenerative diseases especially in neurodegenaration. For instance, the redox-active Fe (II) reacts with H_2O_2 to generate the highly reactive •OH (hydroxyl radical) via the Fenton reaction. High levels of •OH may lead to the development of various oxidant-induced metabolic disorders. Thus, there is a critical link between Fe(II) concentration and oxidative stress in the human biological environment. The crucial role of iron in promoting oxidative stress suggests that chelating agents such as bioactive peptides that can sequester free redox-active iron from its sites of activity to form nontoxic metal complexes could play an important role in preventing oxidative injury. The capacity of the KBH and the fractions was assessed for their ability to compete with ferrozine for ferrous ions, resulting in reduced absorbance of the ferrozine-Fe (II) complex as shown in Fig. 5.5. As expected, GSH showed the significantly highest ($p<0.05$) iron-chelating activity. The samples exhibited iron-chelating potency to various extents, with fractions containing peptides with the 3-5 and 5-10 kDa fractions showing significantly higher ($p<0.05$) chelating

activity when compared to the KBH and 1-3 kDa fraction. This report is different from that of Girgih, Udenigwe and Aluko (2011) who reported the highest iron chelating activity for hempseed hydrolysate, attributing the effect to additive peptide effects. Samples that have shown some potency to scavenge Fe(II) can also scavenge other bivalent transition metal ions (e.g. Zn^{2+} and Cu^{2+}), and hence would partially inhibit the propagation of lipid peroxidation that forms reactive oxygen radicals. This is because stabilization of metal prooxidants through sequestering inhibits free radical production. In addition, when peptides bind metal ions, the redox cycling capacity which is important for some metal-catalyzed oxidation may change. For instance, in ferritin the less reactive ferric ion (Fe^{3+}) is localized in the polypeptide's cavity, where it nucleates and aggregates to form ferric hydroxide core unable to be converted to the more reactive ferrous species (Fe^{2+}) (Arosio and Levi, 2002). The disruption of iron redox equilibrium leads to a reduction of free Fe^{2+}, thereby preventing the decomposition of hydroperoxides (Xiong, 2010). Peptides with amino acid residues containing phosphorylated hydroxyl side chain groups (serine and threonine) and carboxyl groups (glutamic acid and aspartic acid) are good metal-ion binders (Xiong, 2010). Therefore, the high metal chelating activities of 3-5 and 5-10 kDa fractions may be due to their high contents of glutamic acid, aspartic acid, threonine and histidine. It is probable that hydrolysis of peptide bonds led to enhanced Fe^{2+} binding due to increased concentration of carboxylic (COO–) and amine groups in acidic and basic amino acids (Ajibola, Eleyinmi, & Aluko, 2011). Histidine residues have also shown efficiency in metal chelation because of its imidazole ring (Ajibola et al., 2011; Nam, You, & Kim, 2008). In fact the strong Fe^{2+} and Cu^{2+} binding power of the endogenous dipeptide carnosine in muscle tissue has been attributed to the

histidine residue (Xiong, 2010). Over all, the KBH and its ultrafiltrates showed reduced effectiveness as iron chelator when compared with *Jamapa phaseolin* hydrolysates (81%) (Carrasco-Castilla et al., 2012) and the 97% iron chelating activity that was previously described for pea protein hydrolysates after treatment with thermolysin (Pownall, Udenigwe, & Aluko, 2010).

5.3.6.3 Inhibition of Hydroxyl Radical Formation

It has been shown that protracted and severe oxidative stress in humans can lead to the initiation or promotion of a large number of chronic disease conditions (Du & Gebicki, 2004). Superoxide and hydrogen peroxide conversion as well as metal-catalyzed processes, generate the highly reactive hydroxyl radical (•OH) which can oxidize virtually all organic cell constituents including DNA, lipids and proteins (Du & Gebicki, 2004). Once created, the •OH starts a chain of damages by reacting with target molecules that are vital for normal functioning of the cell, or which can become activated and pass the damage to vital cell components. Protein enzymatic hydrolysates and fractions obtained by using various molecular weight cut-off membranes may contain antioxidant peptides which rendered their protective actions in scavenging radicals. The in vitro •OH scavenging activity of KBH and peptide fractions are shown in Fig. 5.6. GSH, which was used as the standard showed significantly highest ($p<0.05$) ability to inhibit production of •OH. The KBH and peptide fractions showed similar levels of •OH scavenging with no significant difference ($p<0.05$). Based on this result, it does not appear that the observed antioxidant activities were impacted by the hydrophobic residues in the hydrolysate and fractions presented. An antioxidant that works against HO• probably acts by blocking formation of the radical from precursors such as superoxide and hydrogen peroxide or by

chelating the transition metal ions such as Fe(II). Yen & Hsieh (1995) attributed the hydroxyl radical scavenging potential to the combined effects of reducing power, donation of hydrogen atoms and scavenging of active oxygen. However in the current study, •OH scavenging ability of the KBH and its peptide fractions was not consistent with their ability to reduce Fe (III) to Fe (II) and DPPH scavenging.

5.3.6.4 Iron (III) Reducing Power

The reducing power assay is often used to evaluate ability of an antioxidant (e.g. peptides) to donate an electron which can reduce $Fe^{3+/}$ferric cyanide complex to the ferrous form (Yildirim, Mavi, & Kara, 2001). The reducing ability of a compound may serve as a significant indicator of its potential antioxidant activity (Meir, Kanner, Akiri, & Philosoph-Hadas, 1995; Oktay, Gülçin, & Küfreviolu, 2003). Several reports have revealed that there is a direct correlation between antioxidant activities and reducing power of certain bioactive compounds. In particular, assays that establish the ability of various bean protein hydrolysates and their membrane fractions to serve as reducing agents or antioxidants are available in literature (Carrasco-Castilla et al., 2012; Valdez-Ortiz, Fuentes-Gutiérrez, Germán-Báez, Gutiérrez-Dorado, & Medina-Godoy, 2012; Xiong, 2010.; Xu & Chang, 2008; Zhang et al., 2011). During the assay for reducing power, an oxidant probe accepts electron from the antioxidant analyte (e.g. peptides) and becomes converted into the reduced probe which is colored (Berker, Güçlü, Tor, & Apak, 2007). When measured at 700 nm, an increase in absorbance indicates better reducing power of the test sample (Berker et al., 2007). As shown in Fig. 5.5, the KBH and its peptide fractions showed different levels of potency in reducing Fe^{3+} to the ferrous form when compared to the GSH standard. The reducing power of the <1 kDa fraction was the

highest, followed by 5-10 kDa, KBH, then 3-5 and 1-3 kDa, respectively (Fig. 5.7). The reducing power of alcalase-hydrolyzed KBH and its peptide fractions was increased with increasing peptide concentrations. A concentration-dependency of reducing power has also been observed for chickpea protein hydrolysate (Li et al., 2008), *Rumex crispus* L. extracts (Yildirim et al., 2001) and fermented soy protein hydrolysate (Amadou, Le, Shi, & Jin, 2011). The highest activity of the <1 kDa fraction may be due to higher content of the hydrophobic amino acids as evident in Table 5.1. Ajibola et al. (2011) also observed that the 1 kDa fraction from African yam bean seed protein hydrolysate had significantly higher (p<0.05) reducing power when compared to those of hydrolysate and the 1–3, 3–5, 5–10 kDa peptide fractions. These authors and others (Bougatef, 2009) agreed that that smaller size peptides exhibited better reducing power than high molecular weight fractions. However, as reported by Wu et al. (2003) and Pownall et al. (2010) for protein hydrolysates of mackerel and pea seed protein hydrolysate fractions, respectively, the present results relate more to the total hydrophobic amino acid contents of the fractions than the peptide size. Li et al. (2008) reported very similar observation for chickpea protein hydrolysate fractions.

5.3.6.5. Lipid peroxidation inhibition activity

Lipid peroxidation (LPO) is of serious concern to the food industry because it results in subsequent development of undesirable off-flavours, odours, dark colours and potentially toxic reaction (Sakanaka et al., 2004). More importantly to human health is that the free radicals that result from LPO can cause damage to cellular macromolecules through oxidative modifications of the genome, proteins, structural carbohydrates, and lipids, thereby affecting normal physiological functions (Romero et al., 1998). Antioxidants are

capable of inhibiting oxidation of lipids. The demand for natural antioxidants, such as those derived from food, has recently increased because of questions about the long-term safety and negative consumer perception of synthetic antioxidants. The antioxidative activity of various fractions of KBH on the oxidation of linoleic acid is shown in Fig. 5.8. In all the samples, especially in the control, the absorbance increased to a maximum and then decreased gradually. Chen, Muramoto, Yamauchi, & Nokihara (1996) and Chen, Zhao, Zhao, Cong, & Bao (2007) made similar observations for the antioxidant activity of designed peptides and peanut protein hydrolysate, respectively. The decrease in absorbance after the 4^{th} day may be because hydroperoxides are usually unstable and they will gradually decompose into secondary metabolites as the experiment progressed. The products formed during the oxidation reacts with the iron (II) sulphate to form iron (III) sulphate, which in turn will react with ammonium thiocynate to form a colored complex of ferric thiocyanate; therefore, absorption intensity is directly related to degree of linoleic acid oxidation (Chen et al., 1996; Pownall et al., 2010). The data suggest that most of the linoleic acid molecules become oxidized after about four days, which reduces peroxide formation and hence also contributes to decreased absorption as the experiment progressed. When compared with the control, addition of peptides exhibited a noticeable antioxidant property to inhibit the peroxidation of linoleic acid as shown by the reduction in absorption intensity of the incubated sample solutions to various degrees. Generally, the rate of chain reaction initiated during lipid peroxidation is inhibited by the ability of antioxidants to reduce and transform the reactive end products to a more stable form (Sowndhararajan, Siddhuraju, & Manian, 2011). Hydrophobic amino acid residues such as Tyr, Met, His, Lys, and Trp, have been shown to have strong antioxidant activity

against lipid derived-radicals due to the ability of hydrophobic amino acids to interact with the lipids (Chen et al., 1996; Pownall et al., 2010). In the current study, the abilities of the fractions to inhibit the peroxidation of linoleic acid corresponded with the amount of hydrophobic amino acid residues in the peptide fractions. The hydrolysates with higher hydrophobic amino acid residues possessed stronger antioxidant activity because hydrophobicity of the compounds was important for interaction with and protection of the hydrophobic linoleic acid from peroxidation. Consequently, the <1 and 5-10 kDa fractions exhibited the highest inhibitory activity, followed by 3-5 kDa and then 1-3 kDa. Although, the <1 kDa fraction had the highest hydrophobic amino acid residues, histidine containing peptides which was highest in the 5-10 kDa fraction may have also played key role in the inhibition of linoleic acid oxidation. This is because of histidine's ability to trap lipid peroxyradical (Erdmann, Cheung, & Schröder, 2008). GSH showed the highest inhibitory activity in the first five days, after which inhibitory activity was similar to the 1-3 and 5-10 kDa peptides. Pownall et al. (2010) reported similar trend in GSH inhibitory activity when compared with pea protein hydrolysate and its membrane fractions. Samples (1 mg/ml) of pea protein hydrolysate and its high performance liquid chromatography fractions as reported by Pownall et al. (2010), exhibited stronger ability to inhibit linoleic acid oxidation over 7 days than observed for the KBH fractions in the current study. However, peanut protein hydrolysate even at 2mg/ml, exhibited negligible inhibitory activity of linoleic acid peroxidation (Chen et al., 2007) when compared to the observations in our study.

5.4. CONCLUSIONS

The <1 and 5-10 kDa fractions exhibited significantly highest ($p<0.05$) renin inhibition and the ability to scavenge DPPH free radical, inhibit peroxidation of linoleic acid and reduce Fe^{3+} to Fe^{2+}. Therefore, the fractions with <1 and 5-10 kDa peptides showed better potential as antihypertensive and antioxidant peptides, probably due to their slightly higher contents of hydrophobic amino acids. However, it is also well known that smaller size peptides may be more bioactive because of the higher probability for increased rate of intestinal absorption (without structural degradation) and entry into cells when compared with the bigger size peptides.

Table 5.1: Percent amino acid composition of kidney bean hydrolysate (KBH) and peptide fractions derived from the hydrolysis of kidney bean globulins

Sample ID	Globulin	KBH	<1 kDa	1-3 kDa	3-5 kDa	5-10 kDa
ASP	13.6	13.41	10.6	12.97	12.98	16.37
THR	3.57	3.35	3.42	3.24	3.18	3.52
SER	6.83	7.18	8.09	7.49	7.33	6.26
GLU	18.59	18.35	19.35	19.48	19.65	17.58
PRO	3.34	3.8	2.73	3.66	4.25	4.92
GLY	3.71	3.49	3.74	3.57	3.57	3.29
ALA	3.58	3.19	3.67	3.24	3.2	2.51
CYS	0.049	0.14	0.12	0.12	0.2	0.19
VAL	4.57	4.88	4.64	4.43	4.39	4.95
MET	0.62	0.55	0.46	0.55	0.54	0.57
ILE	4.47	4.57	4.47	4.28	4.25	4.24
LEU	9.30	9.02	9.95	8.99	8.76	7.85
TYR	3.87	3.89	4.78	3.87	3.86	2.71
PHE	7.26	6.96	7.27	6.71	6.63	6.76
HIS	3.16	4.16	3.92	4.00	3.98	5.11
LYS	7.29	6.87	7.06	7.05	7.02	6.61
ARG	5.62	5.87	5.42	6.06	5.88	6.16
TRP	0.53	0.34	0.32	0.29	0.34	0.4
HAC*	37.43	36.94	37.37	35.84	36.13	35.68

*HAC: Hydrophobic Amino Acid

Fig. 5.1. Surface hydrophobicity of kidney bean hydrolysate (KBH) and peptide fractions (<1, 1-3, 3-5, & 5-10 kDa) 10 mM phosphate buffer (pH 7.0). Different alphabets indicate significant ($p < 0.05$) differences between different samples.

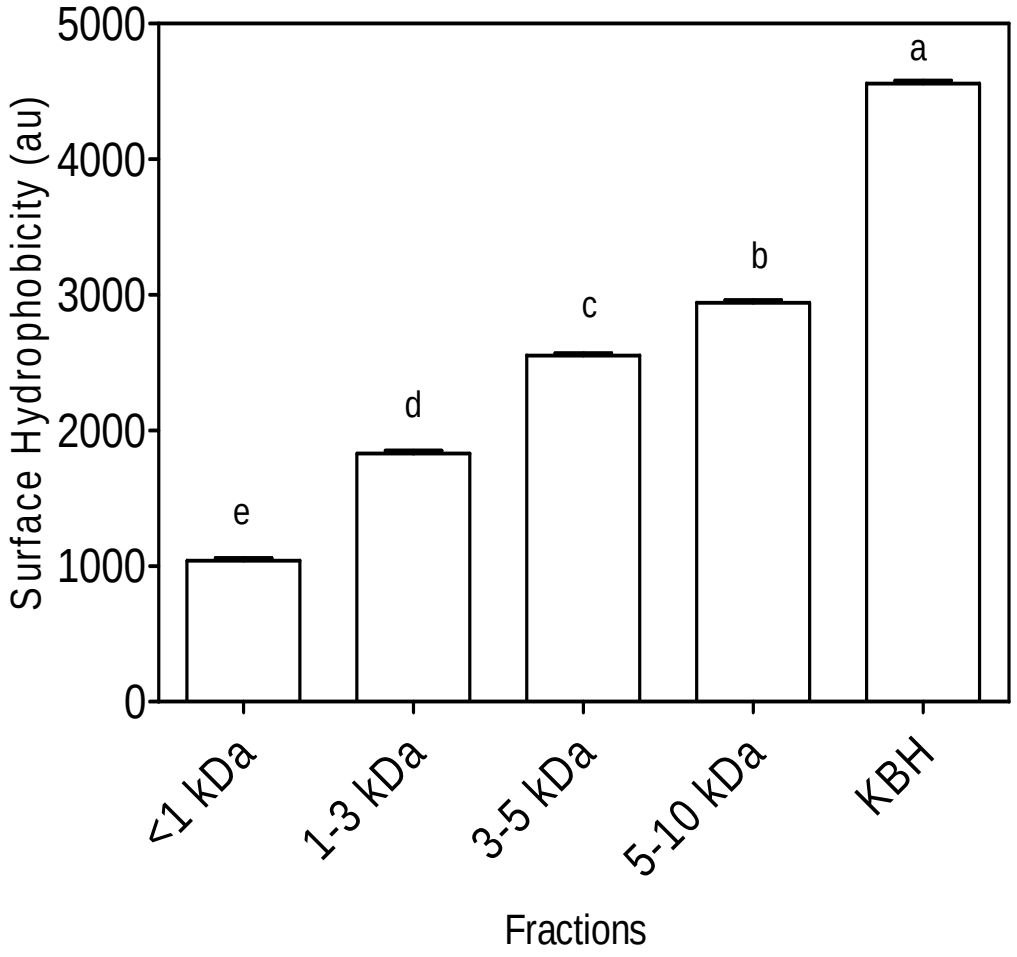


Fig. 5.2. Renin-inhibitory activity of kidney bean hydrolysate (KBH) and peptide fractions (<1, 1-3, 3-5, & 5-10 kDa). Different alphabets indicate significant ($p<0.05$) differences between different samples.

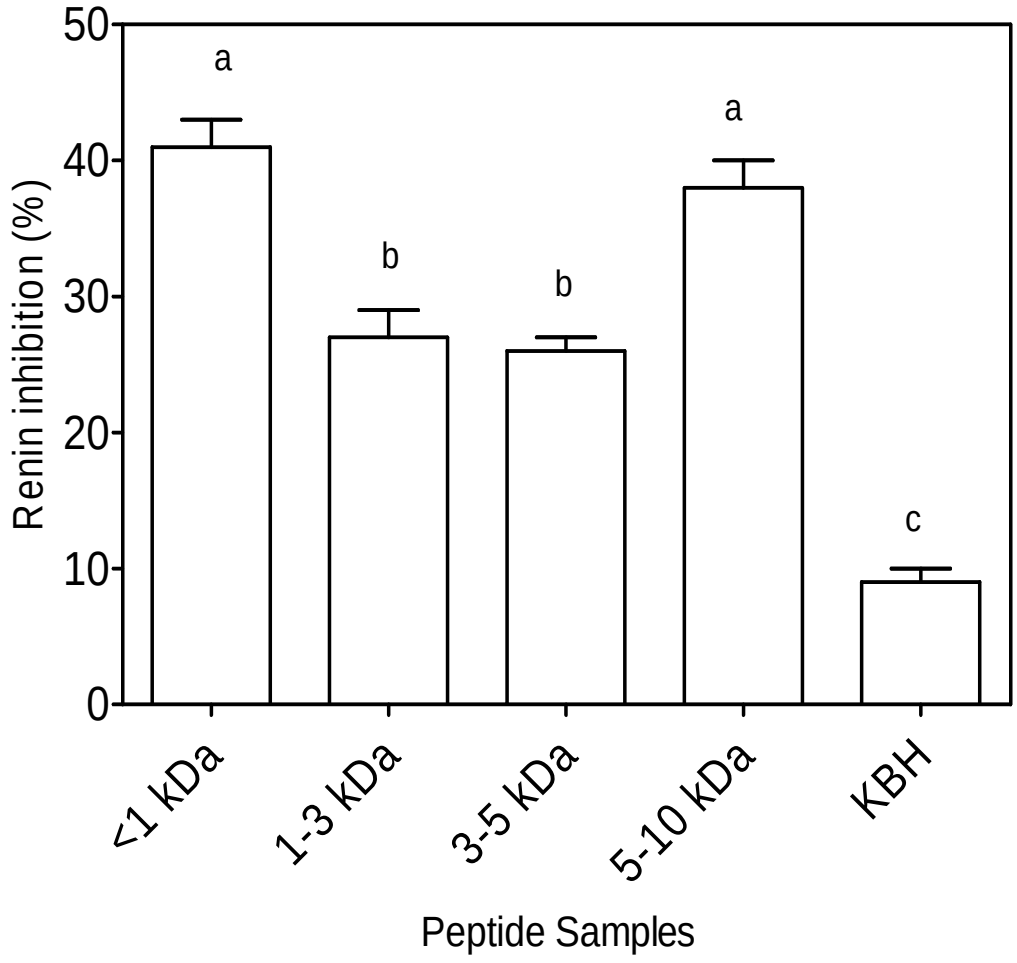


Fig. 5.3. ACE-inhibitory activity (%) of kidney bean hydrolysate (KBH) and peptide fractions (<1, 1-3, 3-5, & 5-10 kDa). Different alphabets indicate significant ($p < 0.05$) differences between different samples.

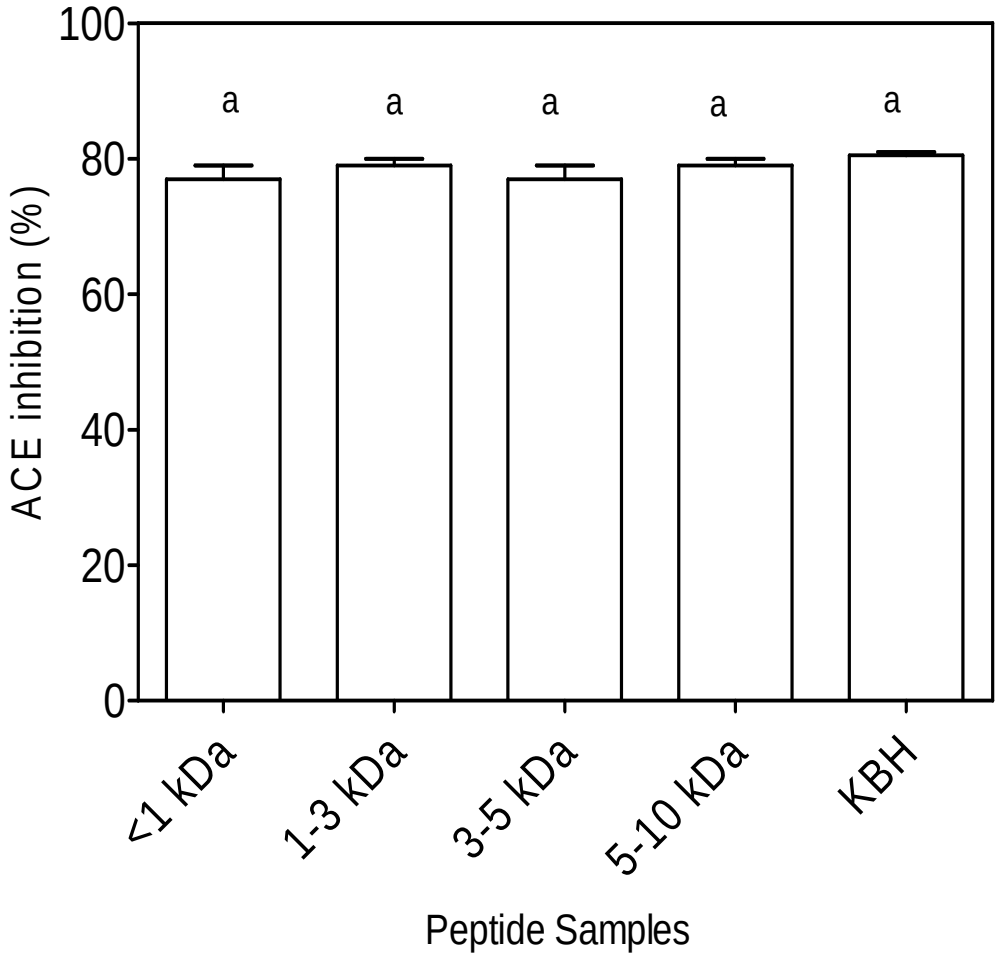


Fig. 5.4. DPPH scavenging activity of 1 mg/ ml of kidney bean hydrolysate (KBH) and peptide fractions (<1, 1-3, 3-5, & 5-10 kDa) with glutathione as a positive control. Different alphabets indicate significant ($p < 0.05$) differences between different samples.

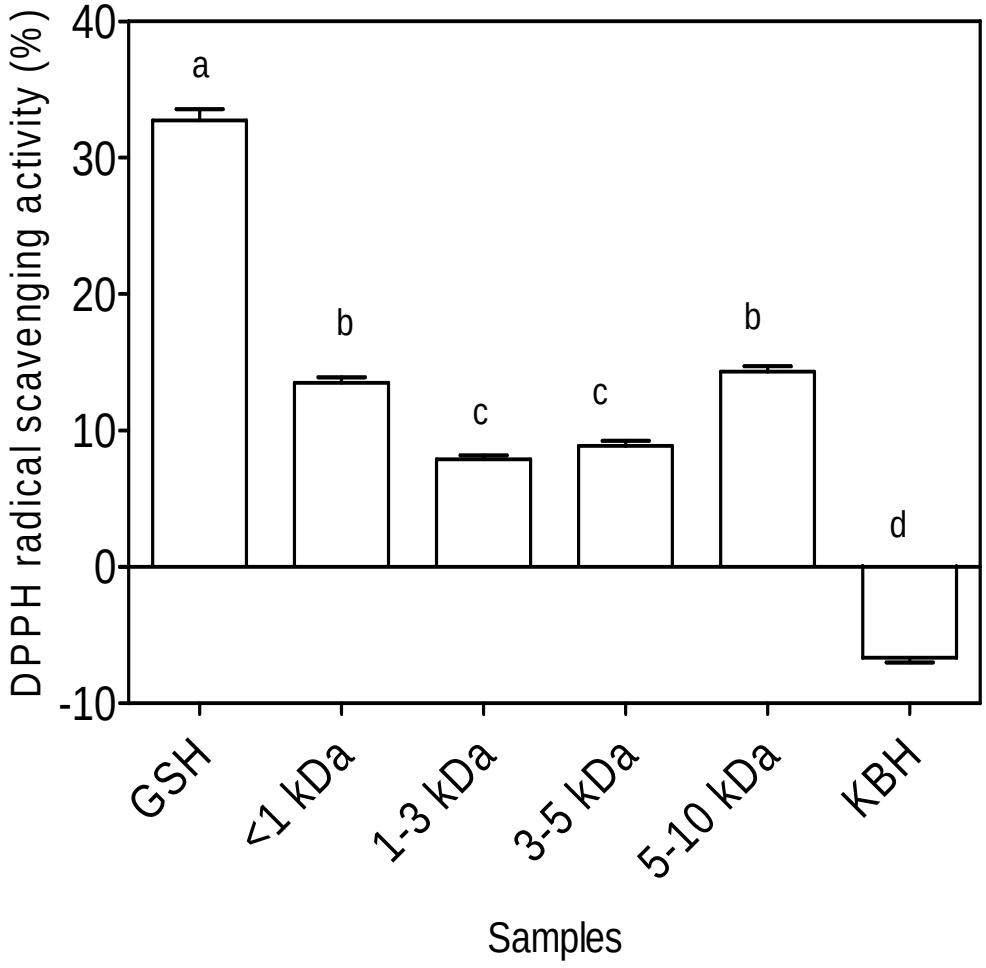


Fig. 5.5. The percentage Fe^{2+} chelation ability of 1 mg/ml of kidney bean hydrolysate (KBH) and peptide fractions (<1, 1-3, 3-5, & 5-10 kDa) with glutathione as a positive control. Different alphabets indicate significant ($p< 0.05$) differences between different samples.

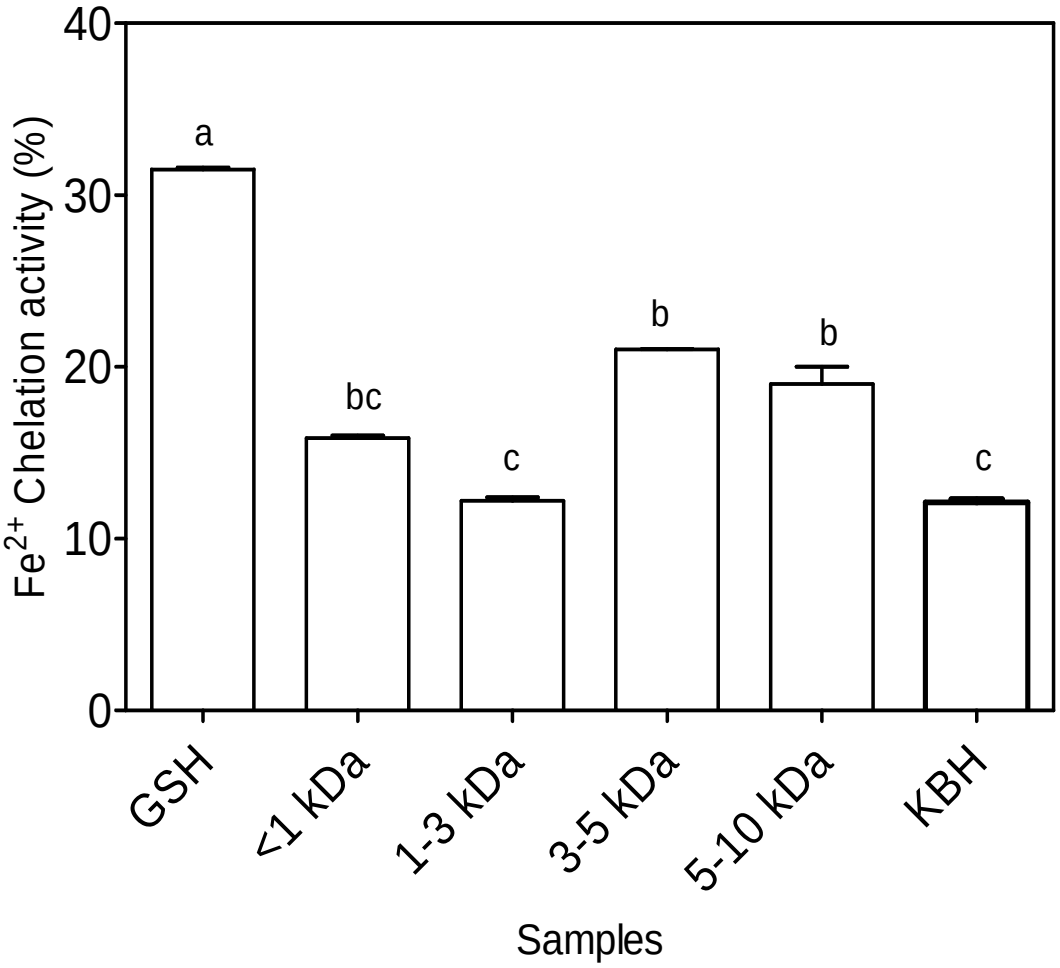


Fig. 5.6. Hydroxyl radical scavenging activity of 1 mg/ ml of kidney bean hydrolysate (KBH) and peptide fractions (<1, 1-3, 3-5, & 5-10 kDa) with glutathione as a positive control. Different alphabets indicate significant ($p < 0.05$) differences between different samples.

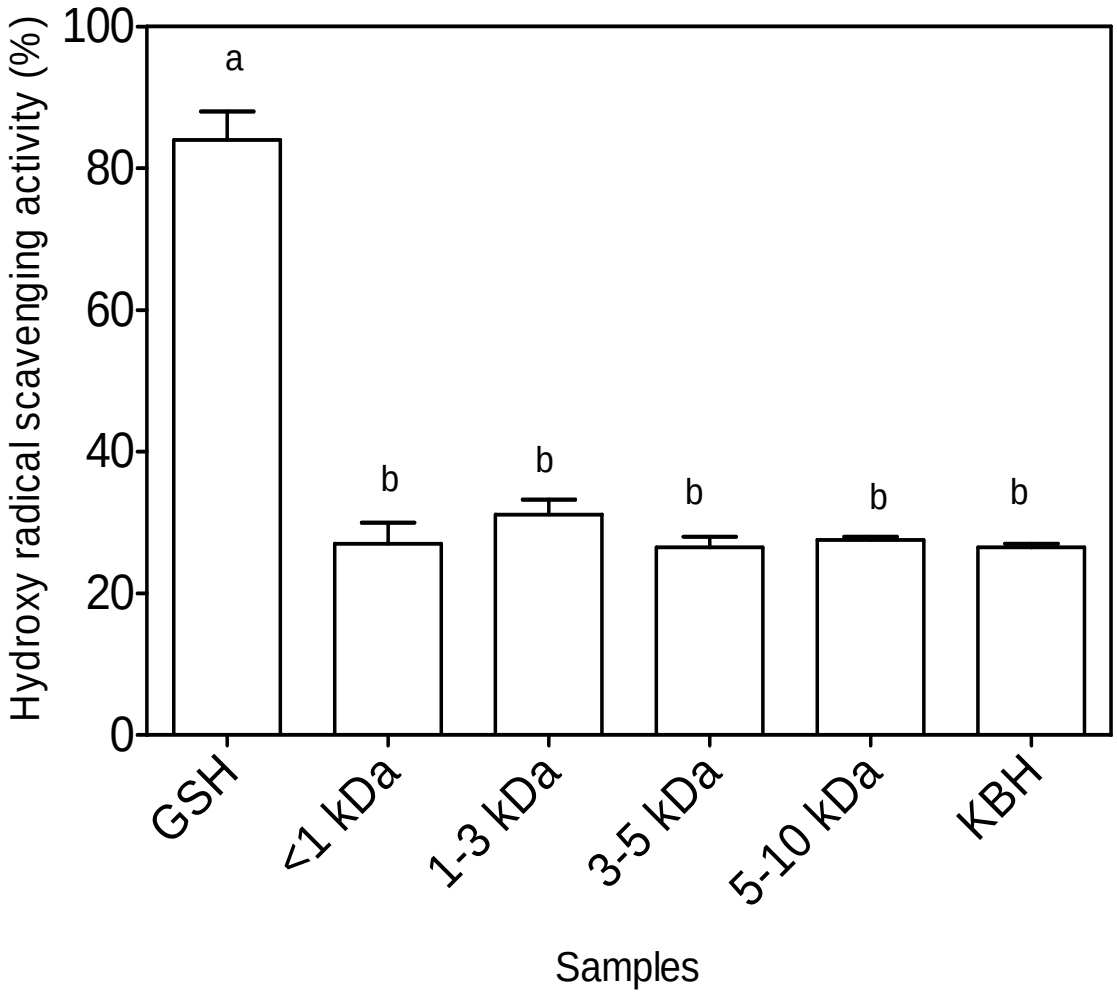


Fig. 5.7. Dose dependent ferric reducing antioxidant property (FRAP) of 1, 5 and 10 mg of KBH and peptide fractions (<1, 1-3, 3-5, & 5-10 kDa).

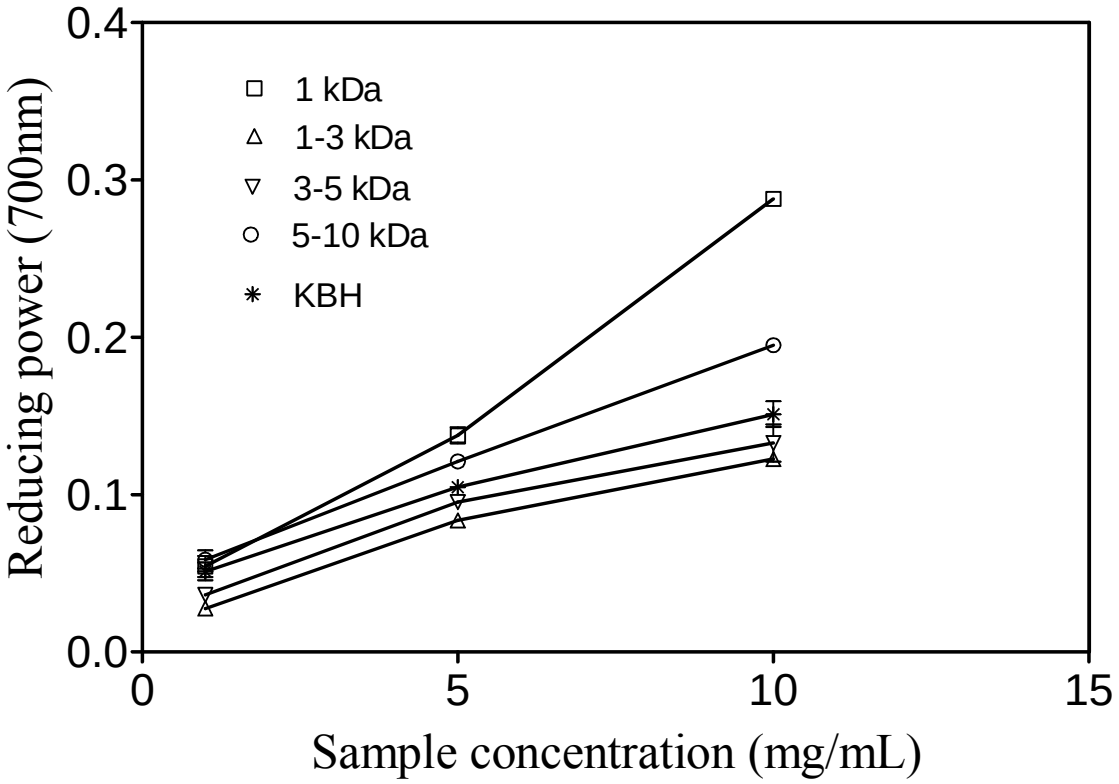
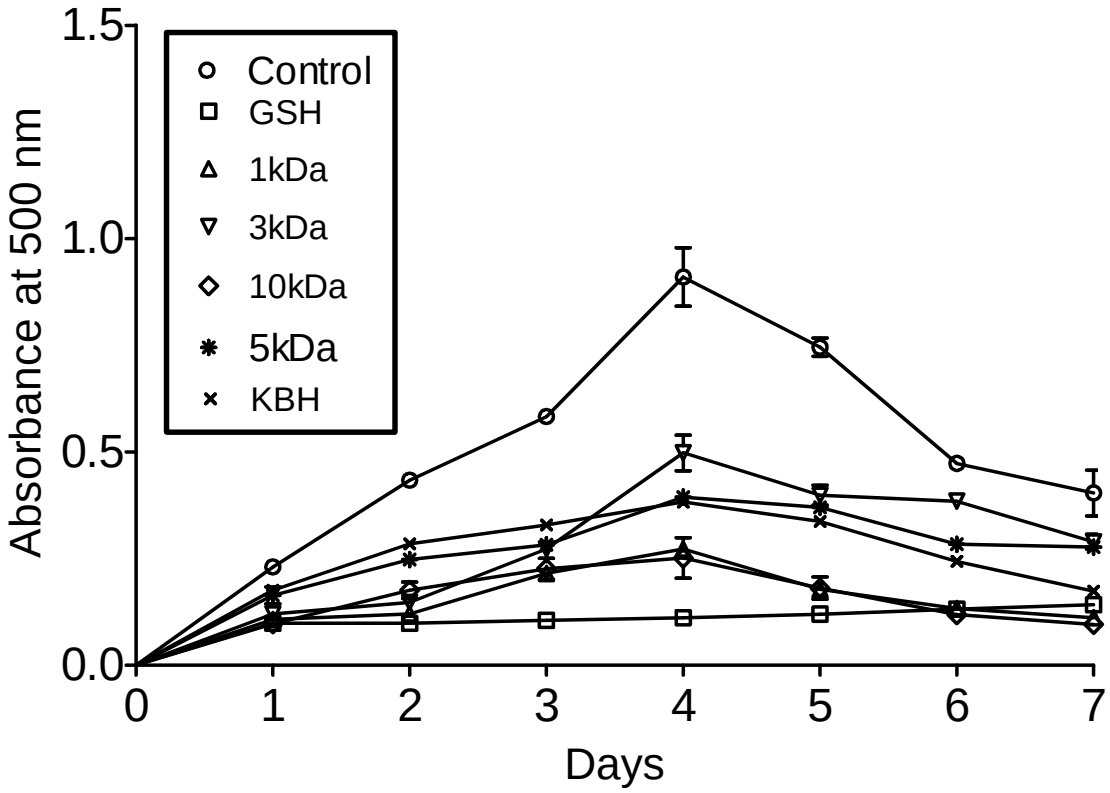


Fig. 5.8. Lipid peroxidation inhibition activity of kidney bean hydrolysate (KBH) and peptide fractions (<1, 1-3, 3-5, & 5-10 kDa) with glutathione as a positive control. Higher absorbance at 500 nm represents higher rate of lipid peroxidation.



References

Ajibola, C. F., Eleyinmi, A. F., & Aluko, R. E. (2011). Kinetics of the inhibition of renin and angiotensin I converting enzyme by polar and non-polar polyphenolic extracts of vernonia amygdalina and gongronema latifolium leaves. *Plant Foods for Human Nutrition, 66*, 320-327.

Aluko, R. E. (2004). The extraction and purification of proteins: An introduction. In R. Y. Yada (Ed.), *Proteins in food processing* ((pp. 323-346).). Cambridge; Boca Raton: Woodhead Pub; CRC Press.

Aluko, R. E. (2008). Determination of nutritional and bioactive properties of peptides in enzymatic pea, chickpea, and mung bean protein hydrolysates. *Journal of AOAC International, 91*, 947-956.

Aluko, R. E., & Monu, E. (2003). Functional and bioactive properties of quinoa seed protein hydrolysates. *Journal of Food Science, 68*, 1254-1258.

Amadou, I., Le, G-W., Shi, Y-H. & Jin, S. (2011). Reducing, radical scavenging, and chelation properties of fermented soy protein meal hydrolysate by lactobacillus plantarum LP6. *International Journal of Food Properties, 14*, 654-665.

Amarowicz, R., & Shahidi, F. (1997). Antioxidant activity of peptide fractions of capelin protein hydrolysates. *Food Chemistry, 58*, 355-359.

Arosio, P. and Levi, S. (2002). Ferritin, iron homeostasis, and oxidative damage. *Free Radical Biology and Medicine 33*, 457-463.

Bamdad, F., Wu, J., & Chen, L. (2011). Effects of enzymatic hydrolysis on molecular structure and antioxidant activity of barley hordein. *Journal of Cereal Science, 54*, 20-28.

Berker, K. I., Güçlü, K., Tor, İ., & Apak, R. (2007). Comparative evaluation of fe(III) reducing power-based antioxidant capacity assays in the presence of phenanthroline, batho-phenanthroline, tripyridyltriazine (FRAP), and ferricyanide reagents. *Talanta, 72*, 1157-1165.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984). Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography - Biomedical Applications, 336*, 93-104.

Bougatef, A., Hajji, M., Balti, R., Lassoued, I., Triki-Ellouz, Y., & Nasri, M. (2009). Antioxidant and free radical-scavenging activities of smooth hound (mustelus mustelus) muscle protein hydrolysates obtained by gastrointestinal proteases. *Food Chemistry, 114*, 1198-1205.

Carrasco-Castilla, J., Hernández-Álvarez, A. J., Jiménez-Martínez, C., Jacinto-Hernández, C., Alaiz, M., Girón-Calle, J., Dávila-Ortiz, G. (2012). Antioxidant and metal chelating activities of phaseolus vulgaris L. var. jamapa protein isolates, phaseolin and lectin hydrolysates. *Food Chemistry, 131*, 1157-1164.

Chen, G-T., Zhao, L., Zhao, L-Y., Cong, T., & Bao, S. (2007). In vitro study on antioxidant activities of peanut protein hydrolysate. *Journal of the Science of Food and Agriculture, 87*, 357-362.

Chen, H-M., Muramoto, K., Yamauchi, F., & Nokihara, K. (1996). Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. *Journal of Agricultural and Food Chemistry, 44*, 2619-2623.

Cheung, H. S., Wang, F. L., Ondetti, M. A., Sabo, E. F., & Cushman, D. W. (1980). Binding of peptide substrates and inhibitors of angiotensin-converting enzyme.

importance of the COOH-terminal dipeptide sequence. *Journal of Biological Chemistry, 255*, 401-407.

Cheung, I. W. Y., Cheung, L. K. Y., Tan, N. Y., & Li-Chan, E. C. Y. (2012). The role of molecular size in antioxidant activity of peptide fractions from pacific hake (merluccius productus) hydrolysates. *Food Chemistry,* 134, 1297–1306.

Corrêa, A. P. F., Daroit, D. J., Coelho, J., Meira, S. M., Lopes, F. C., Segalin, J., Brandelli, A. (2011). Antioxidant, antihypertensive and antimicrobial properties of ovine milk caseinate hydrolyzed with a microbial protease. *Journal of the Science of Food and Agriculture, 91*, 2247-2254.

Cumby, N., Zhong, Y., Naczk, M., & Shahidi, F. (2008). Antioxidant activity and water-holding capacity of canola protein hydrolysates. *Food Chemistry, 109*, 144-148.

Du, J., & Gebicki, J. M. (2004). Proteins are major initial cell targets of hydroxyl free radicals. *International Journal of Biochemistry and Cell Biology, 36*, 2334-2343.

Erdmann, K., Cheung, B. W. Y., & Schröder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *The Journal of Nutritional Biochemistry, 19*, 643-654.

FAO/WHO (1985). Energy and protein requirements. Report of a Joint FAO/WHO/UNU Expert Panel Consultation (Vol. 724). Geneva: World Health Organization

Gehrke, C. W., Wall Sr., L. L., & Absheer, J. S. (1985). Sample preparation for chromatography of amino acids: Acid hydrolysis of proteins. *Journal of the Association of Official Analytical Chemists, 68*, 811-821.

Gibbs, B. F., Zougman, A., Masse, R., & Mulligan, C. (2004). Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. *Food Research International, 37*, 123-131.

Girgih, A.T., Udenigwe, C.C. and Aluko, R.E. (2011). In vitro antioxidant properties of hemp seed (Cannabis sativa L.) protein hydrolysate fractions. *88*, 381-389.

Hartmann, R., & Meisel, H. (2007). Food-derived peptides with biological activity: From research to food applications. *Current Opinion in Biotechnology, 18*, 163-169.

Hayakawa, S., & Nakai, S. (1985). Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins. *Journal of Food Science, 50*, 486-491.

Hooper, N. M. (1991). Angiotensin converting enzyme: Implications from molecular biology for its physiological functions. *International Journal of Biochemistry, 23*, 641-647.

Hou, W.C., M.H. Lee, H.J. Chen, W.L. Liang, C.H. Han, Y.W. Liu, & Y.H. Lin. 2001. Antioxidant activities of dioscorin, the tuber storage protein of yam (*Dioscorea batatas* Decne). *Journal of Agricultural and Food Chemistry* 49: 4956-4960.

Jang, J., Jeong, S., Kim, J., Lee, Y., Ju, Y., & Lee, J. (2011). Characterisation of a new antihypertensive angiotensin I-converting enzyme inhibitory peptide from pleurotus cornucopiae. *Food Chemistry, 127*, 412-418.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. applications of bioprocesses used in isolation and recovery *Current Pharmaceutical Design, 9*, 1309-1323.

Kodera, T., & Nio, N. (2006). Identification of an anqiotensin I-converting enzyme inhibitory peptides from protein hydrolysates by a soybean protease and the

antihypertensive effects of hydrolysates in spontaneously hypertensive model rats. *Journal of Food Science, 71*, C164-C173.

Landry, J., & Delhaye, S. (1992). Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. *Journal of Agricultural and Food Chemistry, 40*, 776-779.

Li, H., & Aluko, R. E. (2010). Identification and inhibitory properties of multifunctional peptides from pea protein hydrolysate. *Journal of Agricultural and Food Chemistry, 58*, 11471-11476.

Li, Y., Jiang, B., Zhang, T., Mu, W., & Liu, J. (2008). Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). *Food Chemistry, 106*, 444-450.

Li, Y., Liu, J., Zhang, T., Jiang, B., & Mu, W. (2008). Enzymolysis technology optimization for production of antioxidant peptides from chickpea protein. *Nongye Gongcheng Xuebao/Transactions of the Chinese Society of Agricultural Engineering, 24*, 268-273.

Li, Y., Li, B., He, J., & Qian, P. (2011). Quantitative structure–activity relationship study of antioxidative peptide by using different sets of amino acids descriptors. *Journal of Molecular Structure, 998*, 53-61.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978). A modification of the lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry, 87*, 206-210.

Matsui, T., & Matsumoto, K. (2006). Antihypertensive peptides from natural resources . In T. H. M. Khan, & A. Ather (Eds.), *Lead molecules from natural products: Discovery and new trends* (pp. 255-271). Amsterdam: Elsevier.

Matsui, T., & Tanaka, M. (2010). Antihypertensive peptides and their underlying mechanisms. In Y. Mine (Ed.), *Bioactive proteins and peptides as functional foods and nutraceuticals* (1st ed., pp. 43-53) Wiley-Blackwell,.

Medina-Godoy, S., Ambriz-Pérez, D. L., Fuentes-Gutiérrez, C. I., Germán-Báez, L. J., Gutiérrez-Dorado, R., Reyes-Moreno, C., & Valdez-Ortiz, A. (2012). Angiotensin-converting enzyme inhibitory and antioxidative activities and functional characterization of protein hydrolysates of hard-to-cook chickpeas. *Journal of the Science of Food and Agriculture,* Retrieved from www.scopus.com

Megías, C., Pedroche, J., Yust, M. M., Julio Girón-Calle, M. A., Millán, F., & Vioque, J. (2007). Affinity purification of copper chelating peptides from chickpea protein hydrolysates. *Journal of Agricultural and Food Chemistry, 55*, 3949-3954.

Meir, S., Kanner, J., Akiri, B., & Philosoph-Hadas, S. (1995). Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves. *Journal of Agricultural and Food Chemistry, 43*, 1813-1819.

Mine, Y., Li - Chan, E. C. Y., & Jiang, B. (2010). Biologically active food proteins and peptides in health: An overview. In Y. Mine, E. C. Y. Li - Chan & B. Jiang (Eds.), *Bioactive proteins and peptides as functional foods and nutraceuticals* (1st Edition ed., pp. 5-12) Blackwell Publishing Ltd. and Institute of Food Technologists.

Molina Ortiz, S. E., & Wagner, J. R. (2002). Hydrolysates of native and modified soy protein isolates: Structural characteristics, solubility and foaming properties. *Food Research International, 35*, 511-518.

Möller, N. P., Scholz-Ahrens, K. E., Roos, N., & Schrezenmeir, J. (2008). Bioactive peptides and proteins from foods: Indication for health effects. *European Journal of Nutrition, 47*, 171-182.

Morita, A., Kimura, M., & Itokawa, Y. (1994). The effect of aging on the mineral status of female mice. *Biological Trace Element Research, 42*, 165-177.

Nakahara, T., Sano, A., Yamaguchi, H., Sugimoto, K., Chikata, H., Kinoshita, E., & Uchida, R. (2010). Antihypertensive effect of peptide-enriched soy sauce-like seasoning and identification of its angiotensin I-converting enzyme inhibitory substances. *Journal of Agricultural and Food Chemistry, 58*, 821-827.

Nam, K. A., You, S. G., & Kim, S. M. (2008). Molecular and physical characteristics of squid (todarodes pacificus) skin collagens and biological properties of their enzymatic hydrolysates. *Journal of Food Science, 73*, C249-C255.

Nazeer, R. A., & Srividhya, T. S. (2011). Antioxidant peptides from the protein hydrolysates of conus betulinus. *International Journal of Peptide Research and Therapeutics, 17*, 231-237.

Nigel M., H. (1991). Angiotensin converting enzyme: Implications from molecular biology for its physiological functions. *International Journal of Biochemistry, 23*, 641-647.

Oktay, M., Gülçin, I., & Küfreviolu, Ö. I. (2003). Determination of in vitro antioxidant activity of fennel (foeniculum vulgare) seed extracts. *LWT - Food Science and Technology, 36*, 263-271.

Peñta-Ramos, E. A., & Xiong, Y. L. (2002). Antioxidant activity of soy protein hydrolysates in a liposomal system. *Journal of Food Science, 67*, 2952-2956.

Pownall, T. L., Udenigwe, C. C., & Aluko, R. E. (2010). Amino acid composition and antioxidant properties of pea seed (pisum sativum L.) enzymatic protein hydrolysate fractions. *Journal of Agricultural and Food Chemistry, 58*, 4712-4718.

Romero, F. J., Bosch-Morell, F., Romero, M. J., Jareño, E. J., Romero, B., Marín, N., & Romá, J. (1998). Lipid peroxidation products and antioxidants in human disease. *Environmental Health Perspectives, 106*, 1229-1234.

Ruiz-Ruiz, J., Dávila-Ortíz, G., Chel-Guerrero, L., & Betancur-Ancona, D. (2012). Angiotensin I-Converting Enzyme Inhibitory and Antioxidant Peptide Fractions From Hard-To-Cook Bean Enzymatic Hydrolysates. *Journal of Food Biochemistry,* Retrieved from www.scopus.com

Sakanaka, S., Tachibana, Y., Ishihara, N., & Juneja, L. R. (2004). Antioxidant activity of egg-yolk protein hydrolysates in a linoleic acid oxidation system. *Food Chemistry, 86*, 99-103.

Sowndhararajan, K., Siddhuraju, P., & Manian, S. (2011). Antioxidant activity of the differentially processed seeds of jack bean (*Canavalia ensiformis* L. DC). *Food Science and Biotechnology, 20*, 585-591.

Takahashi, S., Takahashi, I., Sato, H., Kubota, Y., Yoshida, S., & Muramatsu, Y. (2001). Age-related changes in the concentrations of major and trace elements in the brain of rats and mice. *Biological Trace Element Research, 80*, 145-158.

Udenigwe, C. C., Lin, Y., Hou, W., & Aluko, R. E. (2009). Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. *Journal of Functional Foods, 1*, 199-207.

Udenigwe, C.C., Li, H. and Aluko, R.E. (2011). Quantitative structure-activity relationship modeling of renin-inhibiting dipeptides. *Amino Acids*. 42, 1379-1386.

Valdez-Ortiz, A., Fuentes-Gutiérrez, C. I., Germán-Báez, L. J., Gutiérrez-Dorado, R., & Medina-Godoy, S. (2012). Protein hydrolysates obtained from azufrado (sulphur yellow) beans (phaseolus vulgaris): Nutritional, ACE-inhibitory and antioxidative characterization. *LWT - Food Science and Technology, 46*, 91-96.

Voronov, S. V., Skirgello, O. E., Troshina, N. N., Orlova, M. A., & Kost, O. A. (2002). A hydrophobic site on the surface of the angiotensin-converting enzyme molecule. *Biochemistry (Moscow), 67*, 553-557.

Wang, J. -., Zhao, M. -., Zhao, Q. -., & Jiang, Y. -. (2007). Antioxidant properties of papain hydrolysates of wheat gluten in different oxidation systems. *Food Chemistry, 101*, 1658-1663.

Wu, J., Aluko, R. E., & Muir, A. D. (2009). Production of angiotensin I-converting enzyme inhibitory peptides from defatted canola meal. *Bioresource Technology, 100*, 5283-5287.

Wu, W., Williams, W. P., Kunkel, M. E., Acton, J. C., Huang, Y., Wardlaw, F. B., & Grimes, L. W. (1996). Amino acid availability and availability-corrected amino acid

score of red kidney beans (phaseolus vulgaris L.). *Journal of Agricultural and Food Chemistry, 44*, 1296-1301.

Wu, W. U., Hettiarachchy, N. S., & Qi, M. (1998). Hydrophobicity, solubility, and emulsifying properties of soy protein peptides prepared by papain modification and ultrafiltration. *Journal of the American Oil Chemists' Society, 75*, 845-850.

Wu, H., Chen, H. and Shiau, C. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (Scomber austriasicus). *Food Research International 36*, 949-957.

Xiong, Y. L. (2010). Antioxidant peptides. In Y. Mine (Ed.), *Bioactive proteins and peptides as functional foods and nutraceuticals* (1st Edition ed., pp. 29-39) Wiley-Blackwell.

Xu, B. J., & Chang, S. K. C. (2008). Total phenolic content and antioxidant properties of eclipse black beans (*Phaseolus vulgaris* L.) as affected by processing methods. *Journal of Food Science, 73*, 19-27.

Yen, G., & Hsieh, P. (1995). Antioxidative activity and scavenging effects on active oxygen of xylose-lysine maillard reaction products. *Journal of the Science of Food and Agriculture, 67*, 415-420.

Yildirim, A., Mavi, A., & Kara, A. A. (2001). Determination of antioxidant and antimicrobial activities of rumex crispus L. extracts. *Journal of Agricultural and Food Chemistry, 49*, 4083-4089.

Yin, S., Tang, C., Yang, X., Wen, Q., & Qi, J. (2011). Surface charge and conformational properties of phaseolin, the major globulin in red kidney bean (*Phaseolus vulgaris* L): Effect of pH. *International Journal of Food Science and Technology, 46*, 1628-1635.

Yuan, L., Wu, J., Aluko, R. E., & Ye, X. (2006). Kinetics of renin inhibition by sodium houttuyfonate analogs. *Bioscience, Biotechnology and Biochemistry,* 70, 2275–2280.

Yust, M. D. M., Millán-Linares, M. D. C., Alcaide-Hidalgo, J. M., Millán, F., & Pedroche, J. (2012). Hypocholesterolaemic and antioxidant activities of chickpea (*Cicer arietinum* L.) protein hydrolysates. *Journal of the Science of Food and Agriculture,* 92, 1994-2001.

Zecca, L., Youdim, M. B. H., Riederer, P., Connor, J. R., & Crichton, R. R. (2004). Iron, brain ageing and neurodegenerative disorders. *Nature Reviews Neuroscience, 5*, 863-873.

Zhang, T., Li, Y., Miao, M., & Jiang, B. (2011). Purification and characterisation of a new antioxidant peptide from chickpea (cicer arietium L.) protein hydrolysates. *Food Chemistry, 128*, 28-33.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Following previously published scientific evidence that recommended increased consumption of pulses from different species to improve health, there is the need to provide new and additional information on value-added utilization of pulse proteins. Therefore, this thesis work explored research that focused on the characterization of functional, physicochemical, and structural properties of red kidney bean proteins. In order to provide new information on potential use of kidney beans as a raw material to produce functional food and nutraceutical ingredients, this work also focused on production and functional characterization of enzymatic protein hydrolysates. Based on a popular proposition that proteins (globulin and albumin) from kidney beans can be used as ingredients in novel food product formulations, data from interdisciplinary research by food technologists, food chemists and nutritionists will advance knowledge on the various processing conditions that may affect the functionality. Monitoring of certain changes (e.g. pH and ionic strength) is important, as they may impact the nutritional and functional properties of the processed food. The optimization of the processing requirements in terms of quality and functionality, besides other factors, such as yield and energy use, will be essential to introducing successfully more value-added pulse processing and the incorporation of these ingredients into foods. Eventually, this will open the door to creating new ingredient markets for pulses, leading to new food products and reformulated food products that address consumer needs.

Our goal in this thesis was to undertake the structural and functional characterization of red kidney bean (*Phaseolus vulgaris*) proteins and enzymatic protein

hydrolysates. The thesis is divided into six chapters in a manuscript format. Chapter 1 reviewed literature from various sources concerning the nature of proteins in pulses and specifically kidney beans, functional properties, structural properties and the role of peptides in hypertension and oxidative stress. The study in chapter 2 was encouraged by previous studies of various functional and physicochemical properties of major proteins in pulse crops, including the globulins and albumins, which indicated good potential for their application in food formulations in view of their excellent functional properties. Because of paucity of information that compares physicochemical and functional properties of albumin and globulin proteins from kidney bean, we were challenged to respond to the research need. Consequently, chapter 2 compared the functional properties of albumin and globulin protein fractions from kidney beans and how their use as ingredients may potentially affect behavior of the food products during and after processing. Our work has revealed that the intrinsic physicochemical and functional properties such as amino acid composition, protein solubility, water holding capacity, foaming capacity, foam stability, emulsion capacity, and emulsion stability may be preferred for the globulin proteins over albumins.

Considering the various food functionality advantages of the globulin proteins as reported in chapter 2, plus the potential commercial viability based on the relatively high yield and the protein contents (compared to the albumin protein), chapter 3 reports on the purification of the globulin protein (vicilin) using fast protein liquid chromatography (FPLC) followed by investigations of the conformational and structural changes. A better understanding of the conformational changes of kidney bean proteins, particularly globulins, under various buffer conditions can enhance their potential utilization as food

ingredients. This is because the structure of a protein can provide important information regarding its function and mechanism of action in foods. During processing events, the conditions in the food environment changes and hence, the protein structure could be affected. The knowledge of changes in temperature, pH, or ionic strength, and their effects on structure-function relationships as well as their control during food preparation is essential. Spectroscopic methods, including circular dichroism (CD) and fluorescence spectroscopy (FS) of proteins can provide sensitive indications of the conformational changes that may occur (at the molecular level) due to changes in the protein environment. CD and FS of proteins provide important structural information concerning the details of the helical content of proteins or the asymmetric environment of aromatic residues. Considering the paucity of information on the effects of environment, chapter 3 reports on studies of the effects of pH alone or in combination with NaCl concentrations on structural conformations of purified kidney bean vicilin using CD and FS. High amounts of α-helix and *β*- pleated sheet conformations of vicilin were observed, which was sensitive to changes in NaCl concentrations when measured at neutral and alkaline pH values. Addition of NaCl reduced the content of β-sheet and α-helix fractions, which was characterized by weak positive and negative bands at 195 nm and at 215 nm transitions, respectively, suggesting salt-induced structural denaturation of the vicilin. The 3-dimensional tertiary structures as observed from the near-UV CD spectra were also affected by shifts in pH conditions and NaCl addition.

In chapter 4, the denaturation event which could be influenced by the thermal properties of the protein was studied. Far-UV CD spectroscopy indicated that initial heating of the vicilin greatly increased β-sheet content up till 60^{o}C from room

temperature, but showed a steady loss in the tertiary structure as temperature was increased further. However, β-sheet structure was still detectable at 80^{o}C. The denaturation temperature (T_d) of vicilin at neutral pH as measured by differential scanning calorimetry (DSC) was 90.31^{o}C. The result showed the highest denaturation temperature (T_d) and enthalpy of denaturation (ΔH) at pH 5.0, which is close to the isoelectric point (pH 4.5) of the kidney bean vicilin. The T_m and ΔH values were decreased at acidic and alkaline pH values as conditions moved away from the isoelectric point. Addition of NaCl helped to stabilize (increased ΔH values) kidney bean vicilin at pH 7 and 9 but not at acidic pH values. It may therefore be concluded that kidney bean vicilin is fully denatured at around 90^{o}C.

Functional peptides are now an important category within the nutraceuticals food sector. Bioactive plant food peptides may help to reduce the worldwide epidemic of chronic diseases that account for 58 million premature deaths annually. This thesis examined the structure-activity relationships of kidney bean protein hydrolysates and constituent amino acids as they relate to antioxidative and antihypertensive properties. Chapter 5 described the production of enzymatic protein hydrolysates from kidney bean globulin protein and membrane separation of the hydrolysate by the use of specific molecular weight cut-offs. The <1 and 5-10 kDa fractions exhibited significantly highest ($p<0.05$) renin inhibition and the ability to scavenge DPPH free radical, inhibit peroxidation of linoleic acid and reduce Fe^{3+} to Fe^{2+}. We therefore concluded that the fractions with <1 and 5-10 kDa peptides showed better potential as antihypertensive and antioxidant peptides, probably due to their slightly higher contents of hydrophobic amino acids. However, it is also well known that smaller size peptides may be more bioactive because of the higher probability

for increased rate of intestinal absorption (without structural degradation) and entry into cells when compared with the bigger size peptides.

In conclusion, this work has shown that kidney bean globulin proteins possess superior functional properties to the albumins in addition to the bean's value as a food-stuff and nutritional source of proteins and essential nutrients. To exploit the nutritional and functional potential of proteins from kidney beans, isolation from the seeds by applying a number of well-known methods before incorporation as ingredients in many foods may be useful in providing excellent functionality. Depending on the final product, the appropriate processing temperature, salt content and pH may be derived from chapter 3 and 4. As new family of antioxidant and antihypertensive peptides emerge, the *in vivo* bioactive activity of kidney bean peptides will increase use of these naturally occurring peptides as potential dietary ingredients to promote human health.

Future work should include use of the isolated or purified kidney bean proteins in actual food formulations such as stabilization of food emulsions (salad dressings and mayonnaise) and replacement of animal fat in meats to manufacture low-fat products. For the protein hydrolysates, it will be necessary for future work to identify and purify active peptides, including elucidation of amino acid sequence. Finally, *in vivo* activities of the protein hydrolysates and peptides need to be determined using appropriate animal disease models such as the spontaneously hypertensive rats that can enab le determination of blood pressure-lowering effects.