ASSESSMENT OF THE ANGIOTENSIN I-CONVERTING ENZYME (ACE) INHIBITORY PROPERTIES OF BEAN PROTEIN HYDROLYSATES

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ACE INHIBITORY PROPERTIES OF BEAN PROTEINS

This thesis is dedicated to my parents, Kejian Chen and Jianzhong Rui, and my best friend, Qi Liu.

Your unconditional love and support have always been my source of inspiration.

Abstract

The prevalence of hypertension during the last two decades has led to an intensive search for novel angiotensin I-converting enzyme (ACE) inhibitory peptides from food protein sources. Dry beans (*Phaseolus vulgaris*) serve as an important protein source in the human diet. However, their physiological and functional properties are poorly understood. Therefore, this thesis was undertaken to investigate the *in vitro* ACE inhibitory activities of nine varieties of dry beans that are largely grown in Canada.

A preliminary study was carried out to compare protein profiles, including composition, thermal properties and molecular structure for protein isolates (PIs) prepared from the nine varieties of dry beans, by using electrophoresis, reverse-phase high-performance liquid chromatography (RP-HPLC), differential scanning calorimetry (DSC), and Fourier transform infrared spectroscopy (FTIR). Results showed that two varieties, namely, cranberry and light red kidney bean PIs had significantly lower denaturation temperatures as well as different electrophoresis profiles and RP-HPLC chromatograms. Additionally, black bean PIs were missing some proteins in the molecular mass (MM) range of 60-97 kDa and had larger amounts of high MM proteins.

In vitro ACE inhibitory activities were investigated for the nine varieties of dry beans. In the first study, protein extracts from the nine varieties of dry bean were subjected to trypsin and *in vitro* gastrointestinal simulation (GIS) digestion. The results demonstrated all hydrolysates exerted ACE inhibitory activities, with hydrolysates produced by *in vitro* GIS digestion presenting significantly higher ($P<0.05$) degree of hydrolysis (DH) values as well as ACE inhibitory activities than that of tryptic digests. Among all the varieties, small red bean and navy bean showed the highest and second highest ACE inhibitory activity, respectively, for both digestion treatments. The results also showed that ACE inhibitory activity of *in vitro* GIS digests of black bean was significantly higher ($P<0.001$) than that of the tryptic digest.

Thus, the three varieties, namely, small red, navy and black beans were selected for subsequent studies to investigate the influence of thermal and different enzymatic treatments on their ACE inhibitory activities. The highest ACE inhibitory activities were obtained for samples hydrolyzed for 100 min, 95 min, and 95 min using Alcalase/papain digestion for heated navy, black and small red bean PIs, respectively. The corresponding half maximal inhibitory concentration (IC_{50}) values were 68 µg protein/mL, 83 µg protein/mL, and 78 µg protein/mL, respectively. Improved ACE inhibitory activities were obtained after *in vitro* GIS hydrolysates in the order of small red bean > navy bean >black bean.

Small red bean hydrolysate, digested by Alcalase/papain and subsequently *in vitro* GIS digestion was further subjected to fractionation to isolate the most active ACE inhibitory peptides. After a three step purification process, including ultrafiltration, gel filtration and preparative reverse phase high performance chromatography (RP-HPLC). Fraction 28 from RP-HPLC was identified as the most active fraction with an IC_{50} value of 19.3 µg protein/mL. Enzymatic kinetics conducted using this fraction demonstrated competitive inhibition with inhibitor dissociation constant (K_i) of 11.6 µg protein/mL. Peptide with sequence PVNNPQIH was identified from this fraction with IC_{50} value of 206.7±3.9µM.

Overall, the results from the current study expand the scientific knowledge of the physicochemical and biochemical properties of the nine dry bean (*Phaseolus vulgaris*) varieties studied. The comparative studies of the *in vitro* ACE inhibitory activities, protein profiles, thermal properties and secondary structures are the first to be reported in the literature to the best of our knowledge. Results from this work provide a solid base for further research on the ACE inhibitory activities of pulses and should be useful for selecting conditions for potential future utilisation of beans as a source of protein hydrolysates and functional ingredients in novel foods targeting hypertension.

Résumé

La prévalence de l'hypertension au cours des deux dernières décennies a mené à une recherche intensive de nouveaux peptides inhibiteurs de l'ACE provenant de protéines alimentaires. Les haricots secs (Phaseolus vulgaris) sont une source de protéines importante dans le régime alimentaire humain. Cependant, leurs propriétés physiologiques et fonctionnelles sont mal connues. Cette thèse a donc été entreprise pour examiner, in vitro, les activités inhibitrices de l'ACE de neuf variétés de haricots secs qui sont en grande partie cultivées au Canada.

Une étude préliminaire a été effectuée pour comparer les profils protéiques, ainsi que la composition, les propriétés thermiques et la structure moléculaire d'isolats de protéines (PI) préparés à partir des neuf variétés de haricots secs, en utilisant l'électrophorèse, la chromatographie liquide haute performance en phase inverse (RP-HPLC), la calorimétrie différentielle à balayage (DSC) et la spectroscopie infrarouge à transformé de Fourier (FTIR). Les résultats ont montré que les PI de deux variétés, à savoir, le haricot canneberge et le haricot rouge clair, avaient des propriétés distinctement différentes dont, des températures de dénaturations significativement inférieures ainsi que des profils électrophorétiques et des chromatogrammes de RP-HPLC différents. De plus, les profils électrophorétiques du PI du haricot noir montraient que ce dernier n'avait pas de protéines de poids moléculaires entre 60 et 90 KDa et avait de plus grandes quantités de protéines à haut poids moléculaire, et que les PI du haricot Navy, du haricot du grand Nord et du haricot noir avaient des profils différents particulièrement dans la région des petits poids moléculaires.

Les activités inhibitrices de l'ACE ont été examinées pour les neuf variétés de haricots secs. Dans la première étude, les extraits de protéines des neuf variétés de haricots secs ont été soumis à la trypsine et la simulation d'une digestion gastro-intestinale (GIS) *in vitro*. Les résultats ont démontré que tous les

hydrolysats avaient des propriétés inhibitrices de l'ACE, et que les hydrolysats produits par la digestion GIS in vitro présentaient un degré d'hydrolyse (DH) et d'activités inhibitrices de l'ACE significativement plus élevés ($P < 0.05$) que les hydrolysats obtenus avec la trypsine seulement. Parmi toutes les variétés, le petit haricot rouge et le haricot blanc ont montré, respectivement, le plus élevé et second plus élevé taux d'activité inhibitrice d'ACE, et ce, pour les deux traitements de digestion. Les résultats ont aussi montré que l'activité inhibitrice de l'ACE des produits de digestion GIS in vitro du haricot noir était significativement plus élevée ($P < 0.001$) que celle de la digestion avec la trypsine.

Ainsi, les trois variétés, à savoir, des petits haricots rouges, les haricots Navy et les haricots noirs, ont été choisies pour des études ultérieures pour examiner l'influence de la température et de différents traitements enzymatiques sur les activités inhibitrices de l'ACE. Les activités inhibitrices de l'ACE les plus élevées ont été obtenues pour des échantillons hydrolysés lors de la digestion avec l'alcalase suivi de la papaïne pour les PI chauffés du Haricot Navy (hydrolyse de 100 minutes) du petit haricot rouge (hydrolyse de 95 minutes) et du petit haricot noir (hydrolyse de 95 minutes). La valeur correspondant à la concentration médiane d'inhibition IC_{50} était de 68, 83 et 78 µg de protéine/ml, respectivement pour les trois haricots susmentionnés. Les meilleures activités inhibitrices d'hydrolysats ont été obtenues après GIS in vitro par le petit haricot rouge, puis, le haricot blanc, et puis, le haricot noir.

L'hydrolysat du petit haricot rouge, digéré par alcalase/papaine et par la suite par la digestion GIS in vitro a été à nouveau soumis au fractionnement pour isoler les peptides inhibiteurs de l'ACE les plus actifs. Après trois étapes de purification comprenant l'ultrafiltration, la filtration sur gel et RP-HPLC, la fraction 28 obtenue après RP-HPLC a été identifiée comme la fraction la plus active avec une valeur d'IC50 de 24.4 µg de protéine/mL. La cinétique enzymatique réalisée avec cette fraction a démontré une inhibition compétitive avec une constante d'inhibition Ki de 14.7 µg/mL.

Les résultats de l'étude actuelle permettent d'étendre la connaissance scientifique des propriétés physicochimiques et biochimiques des neuf variétés de haricots secs (*Phaseolus vulgaris*) étudiées. Les études comparatives des activités inhibitrices de l'ACE, des profils de protéines, des propriétés thermiques et des structures secondaires sont les premières à être rapportées dans la littérature au mieux de notre connaissance. Les résultats de ce travail fournissent une base solide pour de nouvelles recherches sur les activités inhibitrices de l'ACE des légumineuses et devraient être utiles pour choisir des conditions pour de potentielles utilisations futures des haricots comme source d'hydrolysats protéiques et d'ingrédients fonctionnels dans de nouveaux produits alimentaires visant à réduire l'hypertension.

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Contributions of Authors

This research is conducted by Xin Rui and supervised by Dr. Joyce I. Boye, Dr. Benjamin K. Simpson, and Dr. Shiv O. Prasher. Part of the thesis research has been used to prepare several manuscripts for publications and presentations at conferences as follows:

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Rui, X., Boye, J. I., Barbana, C., Simpson, B. K., & Prasher, S. O. (2012) Angiotensin I-converting enzyme inhibitory properties and digestibility of nine varieties of *Phaseolus vulgaris* protein hydrolysates, *Journal of Nutrition & Food Sciences,* 2 (8):156.

Rui, X., Boye, J. I., Simpson, B. K., & Prasher, S. O. (2012) Angiotensin I-converting enzyme inhibitory properties of *Phaseolus vulgaris* bean hydrolysates: Effects of heat treatments and different enzymatic digestions. *Food Research International,* (accepted).

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Rui, X., & Boye, J. I. (2012) Processing of Dry Bean Flours and Fractions. In: Dry Beans and Pulses – Production, Processing and Nutrition. (M. Siddiq and M. A. Uebersax, eds). (pp. 205-234). Ames, IA: Wiley-Blackwell Publishing.

Simpson, B. K., **Rui, X.**, & Jiang, X. J. (2012) Enzyme-Assisted Food Processing, In: Green Technologies in Food Production and Processing. (J. I. Boye and Y. Arcand, eds). (pp. 327-361). New York: Springer Publishing Company.

Simpson, B. K., **Rui, X.**, & Klomklao, S. (2012) Enzymes in Food Processing, In: Food Biochemistry and Food Processing. 2nd Ed. (B. K. Simpson et al., eds). (pp. 312-364) Ames, IA: Wiley-Blackwell Publishing.

Rui, X., Boye, J. I., Ribereau, S., Simpson, B. K., & Prasher, S. O. (2010) Comparative study of the molecular structure and thermal properties of protein isolates prepared from nine *Phaseolus vulgaris* legumes. CIFST/AAFC Conference, May 30th –June 1st, Winnipeg, MB, Canada (poster presentation).

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Rui, X., Boye, J. I., Simpson, B. K., & Prasher, S. O. (2012) Angiotensin I-converting enzyme inhibitory properties of *Phaseolus vulgaris* bean hydrolysates: Effects of thermal and different enzymatic digestion treatments. IFT 2012 Annual Meeting, June 25th–28th, Las Vegas, NV, USA (poster presentation).

Contributions to Knowledge

The present work expands the scientific knowledge of the characteristics of nine major dry bean varieties (*Phaseolus vulgaris*) largely grown in Canada and presents new information on their *in vitro* antihypertensive properties. The specific contributions of the thesis are as follows:

1. The thesis investigated for the first time the thermal properties and secondary structures of protein isolates extracted from nine varieties of dry beans grown in Canada. It is the first time that cranberry and light red kidney bean protein isolates were shown to have distinctive thermal properties compared to the other investigated varieties.
2. The thesis also showed for the first time that all nine varieties of dry beans possessed *in vitro* ACE inhibitory activities after either tryptic or *in vitro* gastrointestinal simulation (GIS) digestion. ACE inhibitory activity was shown to vary depending on bean variety. The thesis clearly demonstrated that digestibility and the extent of hydrolysis affected the *in vitro* ACE inhibitory activities of the beans.
3. For the first time, the relationship between thermal treatment and ACE inhibitory properties of dry beans was investigated. The results demonstrated that thermal treatment facilitated the release of ACE inhibitory peptides from navy and small red bean proteins, but not from black bean. The thesis further showed that varying the enzymatic treatment influenced the *in vitro* ACE inhibitory property of navy, black and small red bean proteins. Among the conditions tested, sequential digestion using Alcalase and papain was determined to be the most suitable for generating ACE inhibitory peptides for all investigated varieties.
4. The thesis considered for the first time an approach to separate the active ACE inhibitory fractions from dry bean proteins. A three-step purification procedure using ultrafiltration, gel filtration and reverse phase high

performance liquid chromatography sequentially was successfully developed and used for the isolation of the active ACE inhibitory fraction from small red bean hydrolysates. Finally, the study determined that the most active bean fraction inhibited ACE in a competitive inhibition mode and contained with a high degree of probability (i.e., based on mass spectrometric studies and library matching studies) peptide fragments containing the following amino acid sequences: PVNNPQIH.

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List of Abbreviations

AACC	American Association of Cereal Chemists
ACE	Angiotensin I–converting enzyme
ACE2	Angiotensin converting enzyme 2
AT_1	Angiotensin II type 1 receptor
AT_2	Angiotensin II type 2 receptor
β-ME	β-mercaptoethanol
'C'	Contender
d.b.	Dry basis
DBP	Diastolic blood pressure
DH	Degree of hydrolysis
DSC	Differential scanning calorimetry
EDTA	Ehylenediaminetetraacetic acid
ESI	Electrospray ionization
FA-PGG	Furanacryloyl-phenyl-glycyl-glycine
FTIR	Fourier transform infrared spectroscopy
GIS	Gastrointestinal simulation
HA	Hippuric acid
HHL	Hippuryl-$_L$-histidyl-$_L$-leucine
LC	Liquid chromatography
MM	Molecular mass
MS	Mass spectrometry
MT	Metric tons
PHA	Phytohaemagglutinin
PI	Protein isolate
RAS	Renin-angiotensin system
RP-HPLC	Reversed-phase high-performance liquid chromatography
'S'	Sanilac

SBP	Systolic blood pressure
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHR	Spontaneously hypertensive rats
'T'	Tendergreen
T_o	onset temperature
T_d	peak transition temperature

Chapter 1: Introduction

1.1 General introduction

Canada is ranked among the top ten largest dry bean exporting countries in the world (Food and Agriculture Organization of the United Nations, 2011). The country's climate and proper soil conditions favor bean cultivation, production and export. The average volume of dry beans produced annually in Canada between 2005 and 2009 was 291,420 tons for an average value of $ 169,509,000 (Food and Agriculture Organization of the United Nations, 2011). The major dry bean species cultivated in Canada is *Phaseolus vulgaris*, with dozens of cultivars, including navy, pinto, black, dark red kidney, light red kidney, cranberry, small red, great northern, and pink beans (Skrypetz, 2000). Dry beans are sometimes considered as "poor man's meat" since they contain high amounts of proteins (20% to 30%, dry weight basis) and are cheaper than muscle foods (Potter & Hotchkiss, 1998; Sathe, 2002). Dry beans form the staple food in some developing and underdeveloped countries such as India, Sri Lanka, Bangladesh; however, in North America (Canada and the US), dry bean proteins are largely underexploited compared to other legume proteins, such as soybean and peanut proteins (Sathe, 2002).

Interest in food derived proteins/peptides beyond their basic nutritional effects has been increasing steadily in recent years (Hernández-Ledesma, et al., 2011). The term bioactive peptide is used to denote a peptide sequences with from 2–50 amino acid residues eliciting one or more of the following physiological benefits, i.e. antimicrobial and antifungal effects, anti-inflammatory effects, blood pressure-lowering effects, cholesterol-lowering effects, antithrombotic effects, mineral absorption enhancement, immunomodulatory effects, antioxidative effects, enzyme inhibitory effects and opioid activity (Rutherfurd-Markwick & Moughan, 2005). In particular, peptides eliciting blood pressure-lowering effects have attracted considerable and significant interests,

due to the notable fact that hypertension affects about 26.4% of the world's adult population, and cardiovascular diseases are among the leading causes of deaths globally (Kearney, et al., 2005, FitzGerald & Murray, 2006).

Angiotensin I–converting enzyme (ACE) (EC 3.4.15.1) is a key enzyme that regulates blood pressure via renin-angiotensin system (RAS) (Chen, et al., 2009; Erdmann, et al., 2008). It catalyzes the degradation of the decapeptide angiotensin I to the potent vasoconstrictor angiotensin II by cleavage of dipeptides at carboxyl-terminal sites. It is also able to degrade bradykinin, a vasodilator, into inactive peptides (Skeggs, et al., 1956; Yang & Erdös, 1967; Yang, et al., 1970). This dual role enables activation of ACE on the overall elevation of blood pressure.

There are available synthetic ACE inhibitors, the uses of which have achieved great success. For instance, captopril was the first ACE inhibitor successfully used in clinical practice (Ondetti, et al., 1977). However, captopril and the other known synthetics, have significant adverse effects such as cough, exanthema, taste alterations, skin rashes, gastric troubles and edema of lips (Vyssoulis, et al., 2001; Torruco-Uco, et al., 2009). Because of the adverse side effects of the synthetic compounds, there is intense interest in the extraction of ACE inhibitors from natural food sources to serve as substitutes or replacements which could curtail the reliance on synthetic compounds. As a result, peptides with ACE inhibitory activities have been characterized from several food protein sources, for example, milk (Contreras, et al., 2009; Chen, et al., 2007; Jiang, et al., 2007), soy (Lo & Li-Chan, 2005; Mallikarjun Gouda, et al., 2006; Wu & Ding, 2002), sunflower (Megías et al., 2004), beef (Jang & Lee, 2005), buckwheat (Ma, et al., 2006), shark meat (Wu, et al., 2008) and peanuts (Quist, et al., 2009). Several studies have been reported on ACE inhibitory peptides from pulses, such as chickpea (Barbana & Boye, 2010; Pedroche, et al., 2002; Yust, et al, 2003), pea (Barbana & Boye, 2010; Vermeirssen, et al., 2004; Vermeirssen, et al., 2005), and

lentil (Boye, et al., 2010; Barbana & Boye, 2011). However, there have been only very few studies on bioactive peptides conducted on dry bean proteins.

1.2 Rationale and Objectives of Study

1.2.1 Rationale

Prevalence of hypertension during the last two decades has led to an intensive search for novel ACE inhibitory peptides from food protein sources as alternatives to the synthetic ones currently in use. Bioactive peptides from food sources are perceived by consumers as more innocuous and without adverse side effects. Thus far, a number of studies have been carried out on ACE inhibitory properties from dietary protein sources, especially from milk and soybean. However, there is a dearth of information on ACE inhibitory properties from dry beans. Dry beans are the second most economically important legume species after soybeans. It serves as staple food in several developing and underdeveloped countries, but dry bean protein properties, especially in terms of their physiological functional properties, are poorly understood. This lack of fundamental studies of dry bean protein physiological properties have limited their applications as value added components in functional foods.

1.2.2 Objectives

The overall objective of this study is to evaluate nine major varieties of Canadian beans (*Phaseolus vulgaris*) for potential ACE inhibitory properties.

The specific objectives of this study are summarized below:

1) To investigate the molecular and thermal properties of the proteins from nine selected varieties of beans using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), reversed-phase high-performance liquid chromatography (RP-HPLC), differential scanning calorimetry (DSC), and Fouriertransform infrared spectroscopy (FTIR) (Chapter 3).

2) To study the ACE inhibitory properties of enzyme-generated protein

hydrolysates from nine varieties of dry beans using various approaches including trypsin digestions and *in vitro* gastrointestinal simulation digestions. To understand the differences of ACE inhibitory activities from each dry bean protein hydrolysate, and to characterize the hydrolysates by using SDS-PAGE, degree of hydrolysis (DH) values and *in vitro* digestibility studies (Chapter 4).

3) To establish optimum reaction conditions for generating ACE inhibitory peptides from three selected dry bean varieties based on different pretreatments (heat or non-heat), different enzyme combinations (Alcalase + Flavourzyme or Alcalase + papain), and stabilities of ACE inhibitory peptides (Chapter 5).

4) To separate and purify potent ACE inhibitory peptides from the best dry bean candidate using procedures such as ultrafiltration, gel filtration and preparative HPLC, and to characterize fractions with potent ACE inhibitory activities with respect to reaction kinetics and peptides sequencing (Chapter 6).

Chapter 2: Literature review

2.1 Dry bean protein profiles

2.1.1 Dry beans

Legumes, after cereals, are the second largest source of human food and animal feed worldwide (Berrios, 2006). Dry beans, also well known as common beans, are perhaps the most important legume species besides soybeans in terms of their economic value (Berrios, 2006). In botanical nomenclature, dry beans belong to the family *Fabaceae* and genus *Phaseolus*. They are highly polymorphic and include over 14,000 cultivars (Sathe, 2002). Examples of some well-known varieties include kidney bean, navy bean, pinto bean, black bean, and cranberry bean (Makri & Doxastakis, 2006).

Cultivation of dry beans can be traced as far back as 800 B.C., when they were firstly domesticated in central and South America. Cultivation then moved northwards throughout Mexico and U.S., and then to other parts of the world (Berrios, 2006). During the past 50 years, global dry bean production has doubled from 11,173,313 metric tons (MT) in year 1961 to 23,229,224 MT in 2010 (Sathe, 2002; Food and Agriculture Organization of United Nations, 2010). The top five dry bean producers in the world in the year of 2009 were Brazil (3,486,760 MT), Myanmar (3,000,000 MT), India (2,430,000 MT), China (1,489,135 MT) and United States (1,150,310 MT) (Food and Agriculture Organization of United Nations, 2009).

The type of dry bean variety varies distinctly geographically and is influenced by climate, dietary habits and historical factors. Kidney beans are the most widely produced and consumed food legume in Africa, India, Latin America and Mexico (Yin et al. 2008). Black beans are popular in Caribbean, South America and Mexico (Pulse Canada, 2011). Large quantities of cluster field beans are grown in southern India (Sreenath, et al. 1996). The top three bean varieties

produced in Canada are navy, pinto and cranberry beans (Skrypetz, 2000).

So far, developing and underdeveloped counties remain the largest consumers of dry beans in the world because of economic reasons (Sathe, 2002). However, dry beans, especially dry bean proteins are underexploited compared to other legume species, such as soybean. Thus, more studies are needed to unveil the beneficial effects of legume proteins to human nutrition, as well as to improve and expand our current knowledge of dry bean proteins.

2.1.2 Dry bean composition

Dry beans are good sources of protein, starch, dietary fiber, vitamins (folic acid and other B-vitamins), minerals (calcium, iron, copper, zinc, potassium, magnesium), and unsaturated free fatty acids (linolenic, linoleic, and oleic acids) (Berrios, 2006; Reyes-Moreno & Paredes-López, 1993). They are also low in fat, sodium and are cholesterol-free.

Dry beans are regarded as “poor man’s meat” due to their high protein content but relatively low cost compared to animal protein foods. Proteins account for 20-30% of the bean flour, which is much higher than that of cereal grains (7-15%). Dry beans contain around 70% of carbohydrates (Potter & Hotchkiss, 1998; Sathe, 2002). The carbohydrates are further categorized based on bean flour as ~40% starch, ~25% dietary fibre, and ~7-10% sugars (Potter & Hotchkiss, 1998; Sathe, 2002; Sosulski & Youngs, 1979; Wang, 2005). The appreciable amounts of dietary fibre in dry beans are associated with several health benefits, such as protection against cardiovascular diseases, diabetes, obesity, and colon cancer (Lee et al. 1992). Dry beans are low in fat. In most bean varieties, fat contents are around 1-3% bean flour, which is much lower than the fat contents of oilseeds; for example, soybean and peanut contain 20-50% fat (Potter & Hotchkiss, 1998).

2.1.3 Dry bean protein classification

The classical Osborne classification has categorized legume proteins into four groups which are albumin (water soluble), globulin (salt soluble), prolamin (alcohol soluble) and glutelin (alkali soluble) (Osborne, 1924). Figure 2-1 presents general extraction steps for separating these four groups of proteins from legume seeds based on their solubility preferences. Besides proteins, dry beans are also reported to contain non-protein nitrogen. Deshpande & Nielsen (1987a) have reported the amount of non-protein nitrogen to be in the range of 8.3 – 14.5% based on total nitrogen contents from 11 varieties of dry beans. Bhatty (1982) also reported dry beans (*Phaseolus vulgaris* cv. Saxa) to contain ~19.1% non-protein nitrogen. Non-protein nitrogen may impact the estimated protein levels of dry beans, and might even cause false results in dry bean digestibility studies (Deshpande & Nielsen, 1987a).

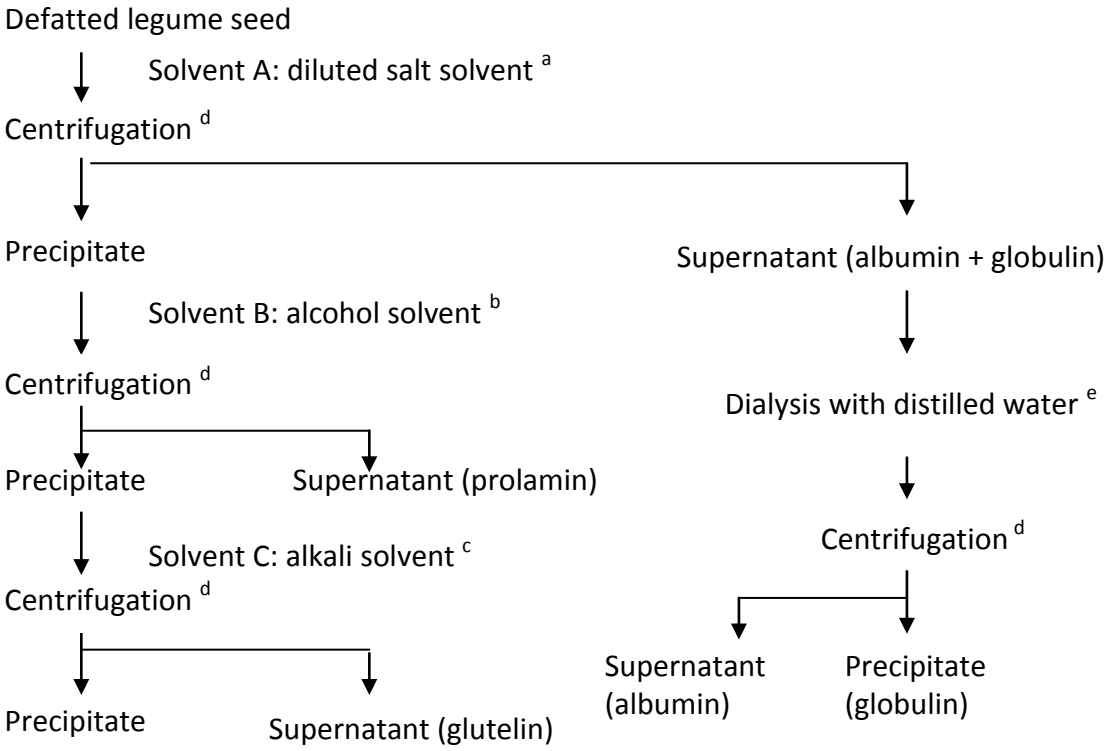


Fig. 2-1. Schematic chart of separating albumin, globulin, prolamin and glutelin.
a:2%–5% NaCl; b: 70% ethanol; c: 0.05 M–0.1 M NaOH; d: centrifugations are performed at 5000 *g* – 12600 *g* for 15 min–20 min at 4–20˚C; e: dialysis is performed at 4˚C; (generated based on modified procedures from Adebowale et al., 2007; Sathe & Venkatachalam, 2007; Subagio, 2006)

Albumin and globulin are widely acknowledged as the major components in dry bean proteins. Combination of water and salt soluble proteins make up 77% - 94% of the protein nitrogen for 11 varieties of dry beans, while alcohol soluble and alkali soluble proteins were found to be in the range of 0.65% - 1.13% and 2.5% - 15.2% (based on protein nitrogen), respectively (Deshpande & Nielsen, 1987a).

Although most studies demonstrated dry beans were composed of a major globulin fraction and a minor albumin fraction, the relative proportions of albumin and globulin seemed to vary or depend on the different varieties. Sathe (1982) reported albumin and globulin fractions constituted 13.0% and 30.5% of dry beans (*Phaseolus vulgaris* cv. Saxa) on a total meal nitrogen basis, respectively. Similarly, great northern bean protein concentrates were reported to contain albumins and globulins at a ratio of 1.00: 1.62 (Satterlee et al., 1975). In another study, navy, great northern, small white, pink, pinto, cranberry, and small red beans were found to contain more globulins than albumins. However, dark red kidney and black bean had albumin: globulin ratios higher than 1, while light red kidney bean had equal amounts of albumin and globulin (Deshpande & Nielsen, 1987a). Discrepancies in the data also exist for non *Phaseolus* beans. For example, moth beans (*Vigna aconitifolia*) contain large quantities of globulins (63.93%, based on total seeds protein) but small quantity of albumins (5.06%, based on total seeds protein) (Sathe & Venkatachalam, 2007). On the contrary, adzuki beans (*Vigna angularis*) has an albumin content of 73.3% compared to 10.4% globulin based on total extractable proteins (Tjahjadi & Breene, 1988). Some researchers have attributed these discrepancies to possible inconsistencies of conditions in the measurements, which is likely due to different ionic strengths and/or buffering capacities produced by different bean flours as well as the interactions of globulins and endogenous tannins (Deshpande & Nielsen, 1987a; Sathe, 1982).

2.1.3.1 Globulin

Globulin acts as a storage protein in beans. Storage proteins are the proteins that accumulate during specific phases of seed development and further degrade to provide nitrogen for new protein synthesis after germination (Derbyshire et al., 1976; Boulter & Croy, 1997). In keeping with this role, globulin is found to be high in nitrogen-rich amino acids, such as arginine, lysine, asparagine and glutamine (Boulter & Croy, 1997). Their high lysine contents make legume proteins a good complement for grain foods (Potter & Hotchkiss, 1998). However, dry bean storage proteins are deficient in sulphur amino acids, possibly due to the low inorganic sulphur content of the usual habitats of dry beans (Boulter & Croy, 1997).

Osborne and Campbell (1898) were the first investigators to describe the two major groups of proteins in globulins and named them as “vicilin” and “legumin” (Osborne & Campbell, 1898). They found these two globulins had slightly different solubility preferences, with legumin being less soluble in dilute salt solutions than vicilin. Additionally, vicilin was more easily coagulated by heat in such solutions. Danielsson (1949) studied sedimentation behaviours of legumin and vicilin by ultracentrifugation, and found legumin and vicilin to have different sedimentation coefficient ($S_{20,\,w}$) of 12.64 and 6.5 – 8.1, respectively. Based on this study, legumin and vicilin were later commonly referred to as 11S globulin and 7S globulin.

Legumin

Legumin is composed of six subunits each with a molecular mass (MM) of around 60 kDa, for a total molecular mass (MM) of around 300 to 400 kDa (Derbyshire et al., 1976). Each subunit can be further disassociated under reducing conditions to a larger acid subunit, or α units (MM: about 40 kDa) and a smaller basic subunit, or β units (MM: about 20 kDa) (Boulter & Croy, 1997).

Legumin is commonly regarded as non-glycosylated, although it might contain up to ~1% of some neutral sugars (Derbyshire et al., 1976; Sathe, 2002).

Vicilin (Phaseolin)

The nomenclature of vicilin in dry beans is complex and sometimes confusing. Vicilin has been variously referred to as phaseolin, globulin-1, glycoprotein II, and α globulin (Osborn & Brown, 1988). However, vicilin and phaseolin are the two names that are frequently used. Thus, we shall use the name phaseolin in our discussion to refer to vicilin from dry beans.

Phaseolin constitutes the major proportion of dry bean storage proteins. In one study, the proportion of phaseolin was reported to be around 88% of the globulin in red bean (*Phaseolus angularis*) (Meng & Ma, 2001a). Another study reported the relative contents of phaseolin to range from 83% to 86% for red bean (*Phaseolus angularis*), red kidney bean (*Phaseolus vulgaris*) and mung beans (*Phaseolus auresus*) (Tang, 2008).

Phaseolin from French bean (*Phaseolus vulgaris,* cv. Tendergreen) has been extensively studied and found to have a trimeric structure with a MM of 150 to 190 kDa, with each subunit having a MM of around 50 kDa (Hall et al., 1977; Boulter & Croy, 1997). Due to different MMs, the three subunits have been named as α, β, and γ subunits corresponding to the largest (51 kDa), second largest (48 kDa) and the smallest subunits (45.5 kDa) (Brown et al., 1981; Hall et al., 1977). Under varied pH conditions, the three subunits reversibly disassociate or associate to different conformations, which are peptides (44 kDa, at pH 12.0), protomers (163 kDa, at pH 7.0), and tetramers of protomers (653 kDa, at pH 3.6) (Sun et al., 1974). Each subunit is glycosylated to different extents which contributes to discrepancies in the molecular weights of the subunits (Paaren et al., 1987). The sugar residues account for around 3% compared to the weight of the proteins (Hall et al., 1977) and are mainly D-mannose and D-glucosamine (Alli et al., 1993; Osborn, 1988).

Gene sequencing techniques provide a clearer picture of the amino acid compositions of these three subunits. Slightom et al. (1985) demonstrated that α, β, and γ subunits were composed of 412, 411, and 397 amino acids, respectively. Studies further showed that the phaseolin subunits were devoid of disulphide bonds, as evidenced by the fact that no dissociation occurred in electrophoretic patterns in the presence of the reducing agent β-mercaptoethanol (β-ME) (Meng & Ma, 2001a; Tang, 2008).

Phaseolin crystals from French bean (*Phaseolus vulgaris*) were first reported in 1990 (Lawrence et al., 1990). The γ subunit with 397 amino acids was composed of two units with similar structures, with each unit containing around 160 amino acids. Each single unit could be further broken down into one α-domain with three helices and one β-domain with a "jelly roll" structure. As a trimer, phaseolin would therefore contain six "α + β" domains arranged in a "disc" with a diameter of 90 Å and a thickness of 35 Å (Lawrence et al., 1990).

Genetic variation of phaseolin was observed from different cultivars of dry beans in terms of subunit compositions. Brown et al. (1981) demonstrated the existence of three types of phaseolins based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) patterns from eleven bean varieties, which were 'Tendergreen' ('T'), 'Sanilac' ('S'), and 'Contender' ('C') named after the typical cultivars. 'T' type phaseolin consists of three subunits (51 kDa, 48 kDa, and 45.5 kDa), whereas 'S' type phaseolin shows distinctive SDS-PAGE profile with five bands at MMs of 49 kDa, 48.5 kDa, 48 kDa, 47 kDa, and 45 kDa. 'C' type phaseolin has an intermediate composition of the two previous types. Studies of phaseolin from 17 varieties of dry beans (*Phaseolus vulgaris*) demonstrated 'T' type phaseolin was obtained for four varieties, which were tendergreen, cranberry, light red kidney and dark red kidney beans, whereas the other 13 varieties had 'S' type phaseolin (Deshpande & Nielsen, 1987b). Phaseolin types were also found to be geographically related. One study screened phaseolin profiles from 106 wild forms and 99 landraces of common

bean (*Phaseolus vulgaris*) from Middle America and the Andean region of South America, and reported most dry beans from Middle America showed 'S' type phaseolin, whereas that from Andean region were mostly 'T' type phaseolin (Gepts et al., 1986). Meanwhile, beans with 'T' type phaseolin were also observed with larger seeds compared to that of 'S' type phaseolin (Gepts et al., 1986). Except 'S', 'T' and 'C' types of phaseolin, phaseolin was observed with some other compositions, such as 'M1–M6' (Romero-Andreas & Bliss, 1985), 'M' (Middle America), 'H' (Huevo de huanchaco), and 'A' (Ayacucho) (Gepts et al., 1986).

2.1.3.2 Albumin

Albumin is a non-storage protein in legume seeds (Millerd, 1975). However, some studies also indicate that albumin may also play a storage protein role (Sauvaire et al., 1984). The molecular mass (MM) of albumin ranges from 11 to 105 kDa in dry beans, with 24 bands in the electrophoretic pattern (Bhatty, 1982). However, albumin from great northern bean showed 14 bands, ranging from 14 to 554 kDa (Sathe & Salunkhe, 1981). Albumin is considered as the most nutritive fraction, based on its high essential amino acids content (Sathe & Venkatachalam, 2007). Studies show that albumins contain higher amounts of methionine and tryptophan compared to the globulin fraction but they possess limited amounts of isoleucine (Bhatty, 1982; Sathe & Venkatachalam, 2007). The albumin fraction also contains many antinutritional factors, such as lectins, trypsin inhibitors and amylase inhibitors.

Lectin (Phytohemagglutinin)

Lectin, also known as phytohemagglutinin, refers to a group of proteins with red blood cells agglutinating activities. The content of lectin in kidney beans (*Phaseolus vulgaris*) has been reported to range from 1 – 10 g/kg based on seeds weight (Nasi et al., 2009). Lectin has high oral toxicity in raw beans. The biological

role of lectin is not clear yet, however, there are studies suggesting possible relatedness to plant defence mechanisms against pathogens and insects (Janen et al., 1976).

Lectin contains two types of subunits: erythroagglutinating phytohemagglutinin (PHA-E) and leukoagglutinating phytohemagglutinin (PHA-L) (Richard et al., 1976). The subunits associate into a tetramer in a random manner and into five different conformations of lectins, namely, L_4, L_3E, L_2E_2, LE_3, and E_4. Tertameric lectin has a MM ranging from 85 kDa to 150 kDa, with each subunit ranging in size from 27 kDa to 37 kDa (Sathe, 2002). Lectins are found both in the albumin and globulin fractions, and are considered as glycoproteins (Osborn & Brown, 1988).

2.1.4 Dry bean protein digestibility

Protein digestibility is important in evaluating protein nutritional quality and biological availability. Compared to animal proteins, dry beans are typically considered to have low protein digestibility, which limits their nutritional values (Sathe, 2002).

Several researchers have attributed the low protein digestibility to phaseolin – the major protein in dry beans. Experimental data show that raw phaseolin is markedly resistant to proteolytic digestion. It was not degraded by pepsin, and was only minimally hydrolyzed by trypsin or chymotrypsin (Bradbear & Boulter, 1984; Liener & Thompson, 1980). However, proteolytic susceptibility of phaseolin was largely improved once it was heated. It is reported that *in vivo* digestibility of phaseolin from navy bean was improved from 57% to 92% after heat treatment (Liener & Thompson, 1980). Proteolytic digestion of heated phaseolin as monitored by SDS-PAGE in time course mode also showed disappearance of almost all the bands on the gels (Deshpande & Nielsen, 1987c).

Many researchers have attributed proteolytic resistance of phaseolin to its compact structure. Phaseolin has a high β structure content and forms a hydrophobic core which is water impenetrable (Bradbear & Boulter, 1984). The

hydrophilic region is found near the center of the phaseolin protein which is easily attacked by proteases, such as trypsin and chymotrypsin, and the structure is easily to be broken into two halves (Nielsen et al., 1988). Upon heating, phaseolin becomes denatured and its tertiary and quaternary conformations are altered which dramatically improves digestibility (Deshpand & Damodaran, 1989a).

Dry bean albumin fraction consists of proteases inhibitors and lectin is also considered to be a contributing factor to the low protein digestibility, although it may not be the dominant one. Unlike phaseolin, heat treatment may decrease protease digestibility of albumin (Genovese & Lajolo, 1996). It has also been reported that the presence of heat-stable trypsin and chymotrypsin inhibitors can slow down the overall rate of hydrolysis or digestibility of salt soluble protein, which is attributed to protein-protein interactions (Deshpande & Nielsen, 1987c). Low digestibility of albumin is considered as multicausal (Genovese & Lajolo, 1996), including factors such as steric hindrance (glycosylation), compact structure (disulfide bonds) and presence of protease inhibitors.

Additionally, non-protein factors, such as polyphenolic compounds and fiber may interact with proteins thus affecting digestibility (Sathe, 2002).

2.1.5 Techniques used in bean protein characterization

2.1.5.1 Electrophoresis

Electrophoresis is one of most useful techniques employed in dry bean protein characterization which based on the analyzes protein/peptides compositions. The main principle of electrophoresis is separation of different molecules, such as protein fractions, by their mobility under an electric field (Boye, 1995). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is one type of electrophoresis used for protein separation based on the size of proteins. A version of the SDS-PAGE procedure involves the use of

β-Mercaptoethanol (β-ME) as reducing agent to break down the disulphide bonds within and/or between protein structures.

Meng & Ma (2001a) carried out SDS-PAGE on red bean (*Phaseolus angularis*) globulin and found that red bean globulin possessed one major band with a MM of 54 to 67 kDa and some minor bands of 29 to 86 kDa in the electrophoretic pattern under non-reducing conditions. SDS-PAGE with and without β-ME has also been performed on protein isolates from red (*Phaseolus angularis*), red kidney (*Phaseolus vulgaris*) and mung (*Phaseolus aureus*) beans. Two major bands with MM of 45 kDa were observed in the electrophoretic patterns under reducing conditions, which was considered as phaseolin. The patterns were unaffected under non-reducing conditions (Tang, 2008).

2.1.5.2 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is used to determine protein thermal properties by calculating differential heat flow transferred between sample and an inert material. Heat differences are recorded as thermograms, in which peaks are generated when deviation of the two heat flows occurs. Peak areas are positively correlated with the enthalpy change (ΔH), and a larger peak indicates a highly ordered protein secondary structure (Koshiyama et al., 1981; Biliaderis, 1983). Additionally, onset temperature (T_o), corresponding to the temperature where the peak starts, and peak transition temperature (T_d), corresponding to the temperature where the peak is at its highest (or lowest) position, reflect the thermal stability of proteins (Ma & Harwalkar, 1988). Peak shapes, either sharp or wide, defined by width at half-height ($\Delta T_{1/2}$), can be related to the cooperativity of protein denaturation during transition (Wright, 1984).

DSC analyses were carried out for vicilins of both hyacinth bean (*Dolichos lablab*) and rice beans (*Phaseolus calcaratus*). Hyacinth bean vicilin showed two overlapping peaks with T_d of 90.90°C and 99.24°C, whereas only one peak was found for rice bean vicilin, with T_d of 91.93°C (Law et al., 2008). Tang

(2008) compared thermal properties of red bean (*Phaseolus angularis*), red kidney bean (*Phaseolus vulgaris*) and mung bean (*Phaseolus aureus*) proteins. It was demonstrated by thermograms from all investigated varieties that only one major endothermic peak was presented, which indicated that the proteins were composed of one predominant component, i.e., vicilin (phaseolin). Thermal stabilities declined, in the following order: red kidney bean (T_d of 90.2°C), red bean (T_d of 87.1°C) and mung bean (T_d of 84.6°C).

2.1.5.3 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) is a technique used to analyze secondary structure of proteins or peptides. A detailed review of the principles of FTIR has been provided by Surewica & Mantsch (1988). Briefly, a spectrum from 1600 to 1700 cm^{-1}, namely "amide I band" is selected for identification of the secondary structure. The amide I band represents the C=O stretching vibration, which is determined by the state of hydrogen bonding between C=O and NH moieties, and further indicates the secondary structure of the protein. In this technique, deuterium oxide solution was used instead of water because the latter absorbs in the infrared region of interest (Surewicz & Mantsch, 1988).

To date, FTIR has been applied for the analysis of the secondary structure of only a few types of beans, namely red bean (*Phaseolus angularis*) (Meng & Ma, 2001b), hyacinth bean (*Dolichos lablab*) and rice bean (*Phaseolus calcaratus*) (Law et al., 2008). The results showed that β-structures, such as β-sheets, β-strands and β-turns, formed the majority of secondary structures in beans compared to α-structures.

2.1.5.4 Reversed-phase high-performance liquid chromatography

Reversed-phase high-performance liquid chromatography *(*RP-HPLC) is a technique applied to legume protein analysis and yields a high resolution and excellent reproducibility. It takes advantage of large-pore spherical silica supports

with hydrophobic ligand columns and polar mobile phases to achieve efficient separations (Pearson et al., 1981).

RP-HPLC has been used for soybean cultivar identification, which showed significant quantitative differences between cultivars (Buehler et al., 1989). Peterson & Wolf (1988) obtained comprehensive results from RP-HPLC chromatogram for soybean protein fraction identification. Their results showed that glycinin elutes out before β-conglycinin and that they share some overlapping parts. RP-HPLC was used in the separation of crystalline protein isolated from white kidney bean (Alli et al., 1983). Five fractions were obtained, and it was determined that the first and second fractions belonged to the α- and β-types of phaseolin, respectively.

2.2 Angiotensin I–converting enzyme (ACE)

2.2.1 Description

The first well known study of Angiotensin I–converting enzyme (ACE) was conducted half a century ago when researchers extracted and partially purified an enzyme from horse plasma, and found that the enzyme had significant effect converting the decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) to the octapeptide angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) (Skeggs, et al., 1956). This is how ACE gained its name. Two decades later, ACE was reported to also cleave the penultimate peptide bond of bradykinin and released a dipeptide Phe-Arg from the carboxyl-terminal end, and inactivating bradykinin in the process (Yang & Erdös, 1967; Yang et al., 1970). The physiological roles of angiotensin II and bradykinin are, respectively, vasoconstriction and vasodilation. Therefore, ACE has a critical dual role in human blood pressure control system. Activation of the enzyme would result in overall elevation of blood pressure.

2.2.1.1 Two isozymes of ACE

There are two molecular forms of ACE present in the human body, namely somatic and germinal (Lanzillo et al., 1985). The somatic form has a larger size (around 150 to 180 kDa), and is located on the endothelial surface of lungs and brush-border membranes of kidneys, intestine, placenta and choroids plexus (Corvol et al., 1995; Erdös & Skidgel, 1986; Eriksson et al., 2002), whereas the germinal form is smaller in size (90 to 100 kDa), and exists only in the testis (Velletri, 1985). Although they have different molecular structures, the two forms of ACE conduct similar functions (El-Dorry et al., 1982; Velletri, 1985).

ACE somatic form from humans contain 4024 nucleotides, encodes 1306 amino acids including a signal peptide of 29 residues at the N-terminus of the polypeptide chain (Soubrier et al., 1988). It comprises of two domains, namely the N- and C-domains, with a junction region linkage between the two domains. The two domains show high similarity of 67% in their deduced amino acid sequences, which indicates that the molecule probably resulted from gene duplication (Soubrier et al., 1988). A similar two domain molecular structure has also been found in mouse (Bernstein et al., 1988), rabbit (Thekkumkara et al., 1992), bovine species (Shai et al., 1992) and rats (Koike et al., 1994).

Human germinal ACE cDNA sequence was reported in 1989 (Lattion et al., 1989; Ehlers et al., 1989). The cDNA sequence comprises of 2477 nucleotides, encodes 732 amino acids, in which 665 amino acids are identical to the C-domain of the somatic ACE. The germinal form contains only one domain, which is the C-domain of the somatic form. This supports the notion that the somatic form results from gene duplication, whereas the germinal form probably represent an ancestral form of the enzyme. Germinal form of ACE is highly hormonal dependable. One paper reported that germinal ACE existed at a low level for immature rats, while a huge rise was observed during puberty (Cushman & Cheung, 1971). However, there was prevention of germinal ACE expression when the pituitary gland was removed from the young rats and no rise of

germinal ACE was observed during puberty (Cushman & Cheung, 1971). Somatic ACE, on the other hand, is present from birth. The biological functions of germinal ACE are not yet clear (Eriksson et al., 2002).

2.2.1.2 The membrane-bound and the soluble forms of ACE

Two different forms of ACE exist in various tissues. The membrane-bound form of ACE is found in the lung and kidney, while the soluble-form of ACE has been purified from plasma, semen, cerebrospinal fluid and other body fluids (Corvol et al., 1995). Both of them have similar molecular weight and identical catalytic functions (Lanzillo, 1985).

The membrane-bound form of ACE exists as an ectoenzyme with its catalytic site exposed to the extracellular milieu (Hooper, 1991). The anchor is specified as the carboxyl terminal with 17 amino acids which interacts with the membrane via hydrophobic interactions (Hooper & Turner, 1987; Soubrier et al., 1988). The soluble form of ACE has dissimilar carboxyl terminal profiles compared with the membrane-bound form (Wei et al., 1991). It seems that mRNA of ACE undergoes different post-translational proteolytic cleavage to produce the two forms (Hooper, 1991). Compared to the membrane-bound form, soluble ACE undergoes cleavage between Arg-1137 and Leu-1138 and deletion of 140 residues at the carboxyl terminal and results in a product without the anchor segment (Beldent et al., 1993). Similarly, the post-translational proteolytic cleavage also takes place in the germinal form of ACE. The cleavage is carried out between sites Arg-663 and Ser-664, which leads to deletion of 74 residues (Ramchandran et al., 1994). The functions of soluble ACE remain unclear (Eriksson et al., 2002).

2.2.1.3 Catalytic site of ACE

ACE is metallopeptidase with zinc as cofactor in its catalytic site (Das & Soffer, 1976). Treatment with metal chelating agents such as ethylenediaminetetraacetic acid (EDTA) causes devastating effects to ACE activity,

whereas the activity could be restored to 40%, 100%, and 160% by addition of ions of manganese, zinc, and cobalt, respectively (Cushman & Cheung, 1971).

Human's cDNA sequence (Soubrier et al., 1988), clearly demonstrated that both N- and C- domains contain the residues involved in the catalytic site, suggesting that both domains contain putative catalytic sites. They were His-361, His-365 and Glu-389 (N- domain) and His-959, His-963 and Glu-987 (C- domain). These results were further supported by observations of somatic ACE from pig kidney and lung which in composed of 2.58 atoms and 2.35 atoms of zinc per molecule, respectively (Williams et al., 1992). Both catalytic sites showed functional properties by expressing either C- or N- domains individually from mutated cDNA in Chinese hamster ovary (CHO) cells (Wei et al., 1991). In this study, both domains functioned independently and both relied on zinc cofactor, although they showed different values of kinetic parameters in hydrolysis of Hippury-Histidyl-Leucine and Angiotensin I, which suggested different susceptibilities to monoanions such as chloride (Wei et al., 1991).

2.2.1.4 Substrate specificity of ACE

ACE, with alternative names of peptidyldipeptidase or dipeptidyl carboxypeptidase, has the major function of releasing dipeptides from carboxyl terminal of peptides. The most well-known reaction conducted with ACE is conversion of the decapeptide Angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) to the octapeptide angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). Other common substrates include the polypeptides Bradykinin, [Met^5]-enkephalins, and [Met^5]-enkephalins-Arg-Gly-Leu. (Skidgel & Erdös, 1987).

Additionally, ACE is also found to cleave tripeptides from carboxyl terminals, when penultimate residue is proline or tryptophan. Examples include substrate such as angiotensin II. ACE also liberates both dipeptide-amide and tripeptide-amide from blocked C terminals (Hooper, 1991). ACE can also react from the N-terminus, and an example of substrate for this type of reaction is with the peptide Glu-His-Trp-Leu-His-Arg-His (Skidgel & Erdös, 1987).

2.2.1.5 Activation of ACE

ACE is primarily activated by monovalent anions, notably chloride. The extent of activation, however, is depended on the types of substrates. Dramatic improvements of reaction rates (i.e., up to 7.5 times) were observed from the addition of chloride ions to the reaction mixture having Angiotensin I, Hippuryl–His–Leu, or synthetic analogue of Angiotensin I Hippuryl-Histidyl-Leucine as substrates (Skeggs et al., 1954; Cushman & Cheung, 1971; Cheung et al., 1980). These substrates which are highly dependent on monovalent anions to induce the reaction are grouped as Class I substrate (Shapiro et al., 1983). Another group of substrates, notably bradykinin, classified as Class II substrates, are hydrolyzed by nonessential activator mechanism, for which a lower degree of enhancement (about 2.8 times) is achieved after addition of chloride anions (Dorer et al., 1974). Substrates belonging to the Class III group also display nonessential activator mechanism; however, compared to Class II substrates, the reaction rates are much lower in the absence of chloride ions, and higher amounts of chloride ions are required to achieve the maximum rate of catalysis (Shapiro et al., 1983).

2.2.1.6 Inhibition of ACE

Cushman & Cheung (1971) have divided ACE inhibitors into four classes, namely: chelating reagents, sulphydryl reagents, heavy-metal ions and peptides. Inhibitors belonging to first three classes act through interaction with zinc ions, the cofactor tightly bounded with ACE. Chelating reagents and sulphydryl reagent are able to chelate the zinc ion, whereas inhibitors of heavy-metal ions are able to exchange with the zinc ion. Inhibitors of the fourth group could be substrates of ACE or other oligopeptides. Ethylenediaminetetraacetic acid (EDTA) and cadmium bromide ($CdBr_2$), belong to chelating agents and heavy metal ions, respectively. Both of them show relatively higher ACE inhibitory activity than the other compounds of the same group, with ACE inhibition of 84% and 51%, respectively at concentration of 10 µM. Furthermore, a pentapeptide from the venom of *Bothrops jararaca*, Pyr-Lys-Try-Ala-Pro (SQ 20,475), belonging to the

fourth inhibitor group also inhibits ACE highly (elicited 59% ACE inhibition at 0.1 μM concentration).

Several predictive models for ACE catalytic sites were developed for understanding the binding mechanism between ACE and inhibitors. The first catalytic site model was established by Ondetti et al. (1977). Researchers assumed that ACE catalytic sites shared similar structure with pancreatic carboxypeptidase A, which was also a zinc-containing metalloprotein. The predictive model of ACE has a positive charged site on one side for binding with terminal carboxyl groups, and a zinc ion on the other side for cutting and hydrolyzing the peptides (Fig.2-2). The distance between the positive charged site and the zinc ion site is longer for the ACE model than that of carboxypeptidase A, as ACE is a dipeptidyl peptidase rather than a peptidase that releases single amino acids.

Some potent inhibitors were generated based on this model, one of which is 2-D-methyl-3-mercaptopropanoyl-L-proline (SQ 14225), which showed outstanding inhibitory properties (IC_{50}: 0.005 μg/mL) and antihypertensive effects *in vivo* (Ondetti et al., 1977). That inhibitor was later renamed captopril and became the first ACE inhibitor antihypertensive drug to be successfully used in clinical practice.

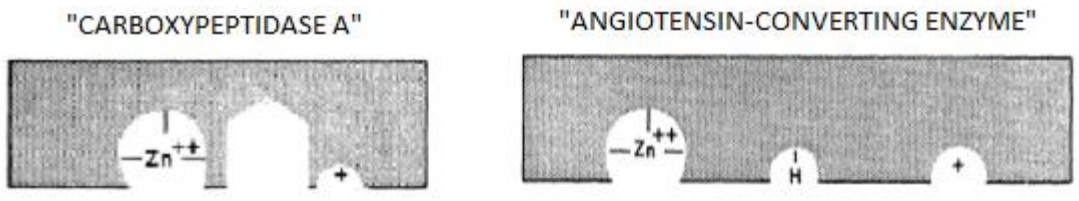


Fig. 2-2. Prediction of catalytic sites for ACE (Modified from Ondetti, et al, 1977)

Five years later, the same research group refined the first model based on the structure of captopril and the venom peptide analogue (Cushman et al., 1982). As shown in Fig.2-3, S1, S1′ and S2′ represented three “subsites” binding

to side chains of antepenultimate, penultimate and terminal amino acids from substrates, and X—H indicates a hydrogen bond donor. (Cushman et al., 1982).

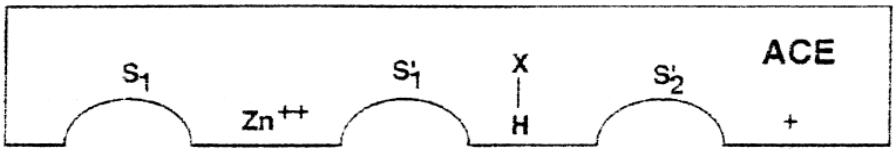


Fig. 2-3. Prediction of catalytic sites for ACE (Modified from Cushman, et al., 1982)

Although the two predicted models described above have contributed enormously to understanding the mode of action and design of novel ACE inhibitors, catalytic sites profiles of ACE remain obscure (Fang et al., 2008). Thus, further studies are required in this field.

2.2.2 Angiotensin I–converting enzyme and hypertension

2.2.2.1 Renin-angiotensin system (RAS)

Blood pressure is a balance of cardiac output and vascular peripheral resistance, which is controlled by complex systems including renin-angiotensin system (RAS), the sympathetic nervous system, kidney and fluid balance mechanisms (Hernández-Ledesma et al., 2011). RAS is a powerful vasoconstriction system in mammals which is not only responsible for maintaining blood pressure levels, but also affects fluid and salt balance via the end product angiotensin II (Eriksson et al., 2002). The main pathway is started by degradation of angiotensinogen, a precursor peptide from the liver, by rennin, and release decapeptide angiotensin I. Angiotensin I is then cleaved to release of the dipeptides at carboxyl-terminal by the action of ACE at the luminal side of the vascular endothelium, to form the octapeptide angiotensin II (Ehlers & Riordan, 1989; Hernández-Ledesma et al., 2011). The physiological function of angiotensin

II is delivered by binding with two receptors, namely, angiotensin II type 1 (AT_1) and angiotensin II type 2 (AT_2) (Eriksson et al., 2002). By directly binding to AT_1 receptors, angiotensin II is able to stimulate aldosterone secretion and induce vasoconstriction, while promoting sodium and water reabsorption in the kidney, and eventually elevating blood pressure (Kovács et al., 2002). However, excessive stimulation of AT_1 receptor will induce a counterbalance mechanism which is delivered by AT_2 receptor suggesting vasodilatation and inhibition of cell growth (Brede et al., 2001; Meffert et al., 1996; Tuner & Hooper, 2002).

Not only does ACE catalyze the conversion of angiotensin I to the vasoconstrictor angiotensin II, it is also able to degrade bradykinin into inactive peptides (Yang & Erdös, 1967; Yang et al., 1970). Bradykinin stimulates bradykinin B_2 receptor to induce the production of nitric oxide (NO) and prostacyclin, which are potent vasodilators (Carretero & Scicli, 1995). Therefore, inhibition of ACE has a dual role in both reducing the formation of angiotensin II as well as preventing degradation of vasodilators, thus leading to an overall blood pressure reduction.

Stimulation of RAS results in an acute blood pressure elevation at circulating level. Additionally, when activated, it could result in long term deleterious effects to the end-organs by inducing growth- and fibrotic-promoting processes on tissue level (Weinberg et al., 2000). In order to achieve effective blockage of RAS on tissue levels, higher ACE inhibitor dose is required, and combinations with other therapies are recommended during the treatment of ACE inhibitor, such as angiotensin II receptor blockers (Weinberg et al., 2000).

Angiotensin converting enzyme 2 (ACE2) has brought new perspectives into classic picture of RAS. ACE2 has an amino acid sequence that partly resembles ACE, and consists of two domains, namely, N and C domains with total molecular mass of around 120 kDa. However, only the N domain has enzymatic activity (Eriksson et al., 2002). The function of ACE2 is different from ACE in that it is a carboxy monopeptidase which is able to cut off carboxyl terminal residue of angiotensin I, to result in angiotensin$_{(1-9)}$, as well as convert angiotensin II to

angiotensin$_{(1-7)}$ (Vickers et al., 2002). Converting of angiotensin$_{(1-9)}$ to angiotensin$_{(1-7)}$ can be effected by ACE. The biological significance of angiotensin$_{(1-7)}$ is not yet clear, however, it has been proposed by some researchers that it has functions as a vasodilator (Turner & Hooper, 2002; Ren et al., 2002). Biological function of ACE2 are further revealed by *in vivo* studies with ACE2-deficient mice that showed pronounced angiotensin II levels, as well as a series of cardiac dysfunctions (Crakower et al., 2002). These symptoms, interestingly, are absent in double mutant mice of both ACE and ACE2, which suggest that ACE2 is rather a negative regulator in RAS (Crakower et al., 2002). A simplified schematic diagram of RAS involves the reactions conducted mainly by ACE and ACE 2 as shown in Fig. 2-4.

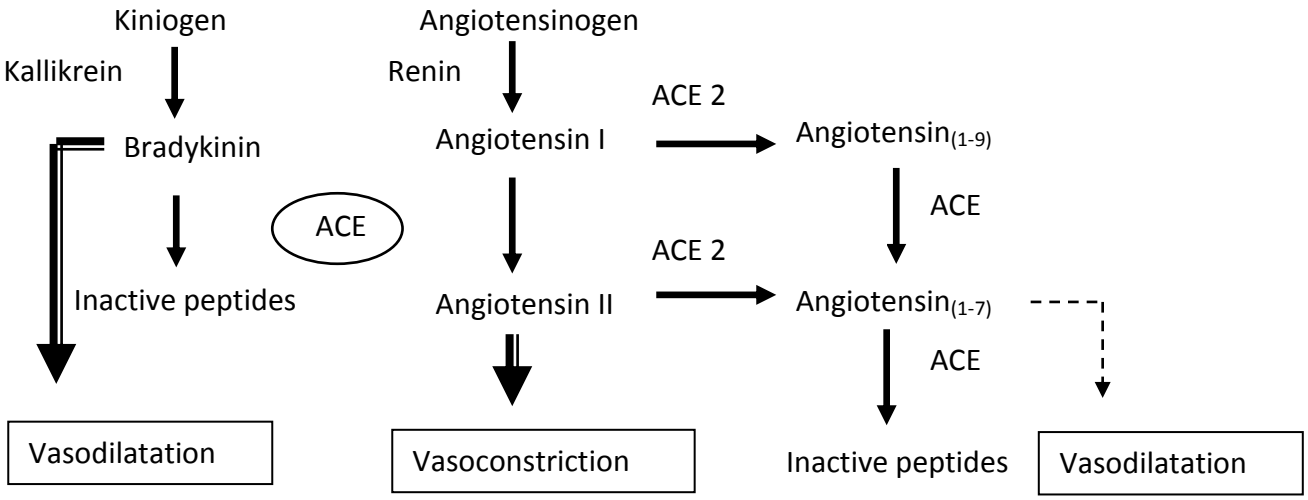


Fig. 2-4. Schematic diagram of the renin-angiotensin system.
(Dashed arrows represented functions that are not confirmed)

2.2.2.2 Traditional ACE inhibitory therapy

As mentioned previously, ACE is known to play an important physiological role in blood pressure control and cardiovascular function, which has led to increased interest in the study and exploitation of ACE inhibitors as potential medical treatments for antihypertensive patients.

Captopril, the first successful ACE inhibitor used in clinical practice, is a

sulphydryl reagent (Cushman et al., 1977). Since that, several drugs have been synthesized such as enalapril, cilazapril, fosinopril, quainapril, and trandolapril. These drugs are now playing important roles in treating hypertension clinically. In 1988, ACE inhibitory therapy was listed as one of the recommended procedures for initial therapy of hypertension by the Joint National Committee on detection, evaluation and treatment of high blood pressure in the United States, as well as three other compounds, namely, diuretic drugs, beta-blockers, and calcium channel blockers (Moser, 1997). ACE inhibitors are found to have similar effectiveness on reduction of blood pressure as beta-blockers but not as effective as diuretic drugs, especially in black people (Materson et al., 1993).

Apart from its anti-hypertension properties, ACE inhibitory therapy provids other protective effects to organs such as kidney and heart. A double-blind study by Lewis et al. (1993) compared the effects of ACE inhibitor (captopril) with a placebo in a randomized trial of insulin dependent diabetes patients. Results demonstrated captopril treatments slowed the progression of diabetic nephropathy, leading to a 50% reduction of combined risks of death, dialysis, and transplantation compared with placebo group. Additionally, captopril is beneficial in decreasing proteinuria. Another random and double blinded study carried out for patients after myocardial infarction showed that patients who were administrated captopril had reduced mortality risk by 19% compared with the placebo group; and the risks of deaths from cardiovascular causes, development of severe heart failure, congestive heart failure and recurrent myocardial infarction were decreased by 21%, 37%, 22% and 25%, respectively (Pfeffer et al., 1992).

However, ACE inhibitory therapy using synthesized compounds might cause several undesirable side effects. Among all the side effects, cough is reported as the commonest. Treating patients with synthesized ACE inhibitors, namely captopril, cilazapril, enalapril, fosinopril, lisinopril, perindopril, quinapril, and trandolapril gave cough symptoms to an average of 9.4% of men out of 650 male

patients and 16.6% of women out of 500 female patients, which led to discontinuation of the treatments for these individuals. Exanthema, gastric troubles as well as edema of lips were also observed for some patients at the ratio of 0.87%, 0.52% and 0.35% (Vyssoulis et al., 2001).

2.3 Angiotensin I–converting enzyme inhibitory peptides

As illustrated in section 2.2.2.2 above, a series of synthetic ACE inhibitors were developed and utilized successfully for blood pressure reduction of hypertensive patients, and found to be effective in treating hypertension. However, those synthetic compounds may also cause adverse side effects, such as cough, exanthema, taste alterations, skin rashes, gastric problems and edema of the lips (Vyssoulis et al., 2001; Torruco-Uco et al., 2009). The associated adverse effects have elicited interests in exploiting ACE inhibitors from natural sources with little or no side effects for use as substitutes for the traditional ACE inhibitory compounds.

Peptides were considered as potential potent ACE inhibitors. In 1960s, extracts of *Bothrops jararaca* venom was found to exert both bradykinin-potentiating activity and ACE inhibitory activity (Ferreira & Silva, 1965; Bakhle, 1968). It was later fractionated and purified into six peptides composed of 9 – 13 amino acids (Ondetti et al., 1971). Studies have reported that many foods derived proteins, when ingested and digested to peptides, might be absorbed through the digestive system and transported into the blood stream. Furthermore, some of the peptides may interact with endogenous enzymes/receptors, and deliver bioactive properties (MartÍnez-Maqueda et al., 2012). Rutherfurd-Markwick & Moughan (2005) reviewed bioactive properties of peptides including antimicrobial, cholesterol-lowering, antithrombotic, immunomodulatory, anti-hypertensive as well as enhancement of mineral absorption. Anti-hypertensive peptides, notably peptides with ACE inhibitory activities were studied from a variety of foodstuffs. Table 2-1 summarizes some recent studies of ACE inhibitory

peptides from different protein sources, with some information on IC_{50} values and peptide sequence.

2.3.1 Angiotensin I–converting enzyme inhibitory peptides from different food sources

Proteins from both animal and plant sources are used as starting materials for producing ACE inhibitory peptides (Table 2-1). Milk and soybean proteins, from animal and plant sources, respectively, are extensively reported to produce bioactive peptides in the literature (Martínez-Maqueda et al., 2012).

There are two major approaches for preparing ACE inhibitory peptides from milk proteins, which are hydrolysis of milk proteins by proteases and milk fermentation (Contreras, et al., 2009). Many peptides with potent ACE inhibitory activities have been prepared from hydrolysates of casein (Contreras et al., 2009; Jiang et al., 2007; Hideaki et al., 1990; Tauzin et al., 2002), whey (Abubakar et al., 1998; Hernández-Ledesma et al., 2007; Vermeirssen et al., 2005) and some of them demonstrated hypotensive effects with animal model systems (Abubakar et al., 1998; Contreras et al., 2009; Hernández-Ledesma et al., 2007). During the manufacture of dairy products, addition of lactic acid bacteria hydrolyzes milk proteins and generates bioactive peptides. Nakamura et al., (1995) reported two ACE inhibitory peptides, VPP and IPP from fermented milk by combination of *Lactobacillus helveticus* and *Saccharomyces cerevisiae*. Maeno et al., (1996) reported that the peptide KVLPVPE liberated from *L. helveticus* fermented milk demonstrated potent antihypertensive activity by oral administration to spontaneously hypertensive rats (SHR). Bioactive peptides were also reported from cheese (Ruiz et al., 2004; Ryhänen et al., 2001).

Soybean protein hydrolysates and fermented soybean products with antihypertensive properties have been reported from several studies. Several enzymes, such as pepsin, pancreatin, protease P, and Alcalase have been applied to digest soy protein for the production of ACE inhibitory peptides (Lo & Li-Chan, 2005; Mallikarjun Gouda et al., 2006; Wu & Ding, 2002). Additionally,

hypotensive effects were also demonstrated with soy sauce, fermented soybean paste, and fermented soybean product (Ahm et al., 2000; Okamoto et al., 1995; Zhang et al., 2006).

A summary of studies on generating antihypertensive peptides from a variety of food proteins is presented in Table 2-1. Recently, antihypertensive properties from pulse protein hydrolysates, i.e., chickpea (Barbana & Boye, 2010; Pedroche et al., 2002; Yust et al, 2003), pea (Barbana & Boye, 2010; Vermeirssen, et al., 2004; Vermeirssen et al., 2005), and lentil (Boye et al., 2010; Barbana & Boye, 2011), were reported. However, few studies have investigated dry bean proteins from this perspective.

Table 2-1 ACE inhibitory peptides from different food sources

Source	Protease applied in hydrolysis	Active Sequence	IC_{50}[a]	Reference
Animal source				
Beef	Thermolysin+proteinase A	VLAQYK	23.2 μg/mL	Jang & Lee, 2005
Bonito muscle	Pepsin	PNRIKYGD HERDPTHIKWGD PTHIKWGD FHERDPTHIKWGD TKTGRSAHVLSRYRPRA KKPTHIKWGD		Hasan et al., 2006
Bullfrog	Alcalase	GAAELPCSADWW	0.95 μM	Qian et al., 2007
Cheese		VRYL KKYNVPQL	24.1 μM 77.1 μM	Ruiz et al., 2004
Human plasma	Trypsin	YLYEIAR	16 μM	Nakagomi et al., 1998
Milk casein	pepsin	RYLGY	0.71 μM	Contreras et al., 2009

		AYFYPEL	6.58 μM	
		YQKFPQY	20.08 μM	
Milk casein	Trypsin	FFVAPFPEVFGK	82 μg/mL	Hideaki et al., 1990
		TTMPLW	13 μg/mL	
		AVPYPQR	13 μg/mL	
Milk casein	Trypsin	FALPQY	4.3 μM	Tauzin et al., 2002
		FALPQYLK		
Milk casein	Neutrase	YQKFPQY		Jiang et al., 2007
		LPQNIPPL		
		SKVLPVPQK		
		LPYPYY		
		FLPYPYY		
Milk casein	*L. helveticus*	KVLPVP	5 μM	Maeno et al., 1996
Milk whey	Protease K	VWPFPG		Abubakar et al., 1998
		GKP		
		IPA		
		FP		
		VWP		
Milk whey	Thermolysin	LQKW	3.5 μM	Hernández-Ledesma
		LLF	82.4 μM	et al., 2007
Milk whey	Pepsin+trypsin+ α-chymotrypsin		0.003 mg/mL	Vermeirssen et al., 2005
Fermented milk		GTW	464.4 μM;	Chen et al., 2007
		GAW	240.0 μM	
Oyster sauce			0.087 mg/mL	Je et al., 2005
Porcine hemoglobin	Pepsin, trypsin, papain separately	VVYPWT		Yu et al., 2006
		LGFPTTKTYFPHF		
Shark meat	Protease SM98011	CF	1.96 μM;	Wu et al., 2008
		EY	2.68 μM;	
		MF	0.92 μM	
		FE	1.45 μM	
Plant source				

Buckwheat	Pepsin+trypsin+chymotrypsin	YQY PSY	4 µM 16 µM	Li et al., 2002
Buckwheat		GPP	23.1 µM	Ma et al., 2006
Broccoli		YPK	10.5 µg/mL	Lee et al., 2006
Chickpea	Alcalase			Pedroche et al., 2002
Chickpea legumin	Alcalase			Yust et al., 2003
Jamapa bean	Alcalase		0.061 mg/mL	Torruco-Uco et al., 2009
	Flavourzyme		0.127 mg/mL	
Lima bean	Alcalase		0.056 mg/mL	
	Flavourzyme		0.0069 mg/mL	
Mung bean	Alcalase	KDYRL VTPALR KLPAGTLF	26.5 µM 82.4 µM 13.4 µM	Li et al., 2006
Pea	Pepsin+trypsin+ α-chymotrypsin		0.016 mg/mL	Vermeirssen et al., 2005
Peanut	Alcalase; Pepsin+pancreatin		8.66 µg/mL 7.93 µg/mL	Quist et al., 2009
Soybean	Alcalase 2.4 L	DLP DG	4.8 µM; 12.3 µM	Wu & Ding, 2002
Soybean	Pepsin+pancreatin		0.13 – 0.93 mg/mL	Lo & Li-Chan, 2005
Soybean 11S globulin	Protease P	VLIVP	1.69 µM	Mallikarjun Gouda et al., 2006
Soy sauce			0.71 – 17.8 mg/mL	Okamoto et al., 1995
Sunflower	Pepsin+pancreatin	FVNPQAGS	6.9 µM	Megías et al., 2004
Wheat gliadin	Pepsin+protease M	IAP	2.7 µM	Motoi & Kodama, 2003

[a] IC_{50} = half maximal inhibitory concentration.

2.3.2 Generating ACE inhibitory peptides via enzymatic treatments

2.3.2.1 Proteases from endogenous system

Endogenous digestive enzymes are extensively utilized for generating ACE inhibitory peptides. The most frequently used proteases are pepsin, trypsin and α-chymotrypsin. Pepsin alone was used to digest ovalbumin (Fujita et al., 2000), bonito muscle (Hasan et al., 2006), and milk casein (Contreras et al., 2009) to generate ACE inhibitory peptides. Trypsin was reported to hydrolyze milk casein (Hideaki et al., 1990; Tauzin et al., 2002), and red lentil (Boye et al., 2010).

Hydrolysis of food protein by combination of digestive enzymes is a common strategy to mimic *in vivo* digestion. By applying pepsin, trypsin, α-chymotrypsin, or pancreatin, proteins undergo similar breakdown as in human digestive system, from which the liberated peptides are expected to be resistant to *in vivo* gastrointestinal digestion (Vermeirssen et al., 2005). A sequential digestion of pepsin and trypsin were utilized for hydrolyzing porcine hemoglobin (Yu et al., 2006); pepsin and pancreatin were employed to digest soy protein (Lo & Li-Chan, 2005), sunflower protein isolates (Megías et al., 2004) and peanut flour (Quist et al., 2009), while pepsin with trypsin and α-chymotrypsin were used for hydrolyzing pea and whey protein (Vermeirssen et al*.,* 2005), and wheat protein (Li et al*.*, 2002).

2.3.2.2 Proteases from microbial sources

Besides endogenous digestive enzymes from animal sources, microbial proteases are also utilized extensively in producing bioactive peptides, such as Alcalase (protease from *Bacillus licheniformis*), Flavourzyme (protease from *Aspergillus oryzae*), thermolysin (protease from *Bacillus thermoproteolyticus*), and neutrase (protease from *Bacillus amyloliquefaciens*).

Alcalase is a broad spectrum protease that is widely used to improve protein functional properties and enhance bioactivities. Torruco-Uco et al., (2009) reported using Alcalase for the production of ACE inhibitory peptides from

jamapa bean protein concentrate. Additionally, Alcalase was used to generate hydrolysates with intensive ACE inhibitory activities from soy protein, chickpea protein, chickpea legumin, and mung bean, with IC_{50} values of 0.065 mg/mL, 0.19 mg/mL, 0.18 mg/mL, 0.64 mg/mL, respectively (Wu & Ding, 2002; Pedroche et al., 2002; Yust et al., 2003; Li et al., 2006).

Flavourzyme is regarded as an exopeptidase, thus it is commonly used individually or in combination with alcalase to produce peptides with smaller sizes (Pedroche et al., 2002). By using Flavourzyme alone, ACE inhibitory activities were observed from jamapa bean and lima bean protein hydrolysates (Torruco-Uco et al., 2009). It has also been reported that Alcalase + Flavourzyme combination yielded antihypertensive hydrolysates from chickpea, pea, and lentil proteins (Barbana & Boye, 2010; 2011).

Thermolysin is a heat stable protease and it mainly acts on peptide bonds near hydrophobic amino acids. Thermolysin digestion of chicken muscle produced hydrolysate with IC_{50} value of 45 μg/mL (Fujita et al., 2000). Thermolysin in combination with proteinase A, were employed to hydrolyze beef protein, and the resulting hydrolysate had a high ACE inhibition activity of 66.4% (Jang & Lee, 2005).

Neutrase has properties similar to thermolysin but is less heat stable. It is reported that neutrase hydrolysates from yak milk casein had the highest ACE inhibitory activity (IC_{50} = 0.38 mg/mL) compared to five other proteases, including trypsin, Alcalase, pepsin, papain, and flavourzyme (Jiang et al., 2007).

Other microbial proteases such as protease A, protease P, and protease M (from *Aspergillus oryae*), produced by Amano Pharmaceutical in Japan, have been applied in several studies to generate ACE inhibitory peptides. Wheat gliadin hydrolysates produced from digestion with pepsin and protease M showed IC_{50} value of 420 μg/mL (Motoi & Kodama, 2003). Protease P was used to digest soybean 11S globulin and produced a hydrolysate with higher ACE

inhibitory activity than those of trypsin, chymotrypsin, and ginger protease digests (Mallikarjun Goudaa et al., 2006).

2.3.2.3 Proteases from plant sources

Plant proteases such as bromelain and papain have also been reported in the literature intended for the hydrolysis of food proteins. Bromelain and papain, from pineapple and papaya respectively, are cysteine proteases, and have been employed to hydrolyze two varieties of lentil proteins, with the papain digests showing higher ACE inhibitory activity than the bromelain digests (Barbana & Boye, 2011). Papain has also been used in chickpea and pea digestions and produced hydrolysates with high potential antihypertensive properties (Barbana & Boye, 2010). Abubakar et al., (1998) reported papain digest from whey protein resulted in high ACE inhibitory activity *in vitro*, but no significant reduction in blood pressure was observed from *in vivo* tests.

2.3.3 Separation and purification of ACE inhibitory peptides

2.3.3.1 Ultrafiltration

Ultrafiltration is the most commonly applied technique as the initial step of purification. In this technique, membrane with specified molecular weight cut off (MWCO) is employed and samples are separated based on their sizes by passing through the membrane. Since ACE inhibitory peptides are mostly small peptides, the use of MWCO of 5 kDa, 3 kDa, and 1 kDa membranes are the most frequently reported in studies. Permeates usually showed higher ACE inhibitory activities than the retentates and are thus collected for further studies. Examples of studies utilizing ultrafiltration as the initial purification step are chickpea protein hydrolysates (Pedroche et al., 2002), pea protein hydrolysates (Vermeirssen et al., 2005), beef hydrolysates (Jang & Lee, 2005), whey hydrolysates (Vermeirssen et al., 2005), mung bean protein hydrolysates (Li et al., 2006), shark meat

hydrolysates (Wu et al., 2008), and milk casein hydrolysates (Contreras et al., 2009).

2.3.3.2 Ion exchange chromatography

It is reported in the literature that ion exchange chromatography is an effective technique for separating and collecting bioactive peptides. Separation of peptides through ion exchange chromatography is related to different charges of the peptides due to varied amino acids compositions. Ion exchange chromatography is usually applied as an intermediate step during purification process. The most frequently used types of columns for collecting ACE inhibitory peptides include SP-Sephadex C-25 cation exchange column (Je et al., 2005; Ma et al., 2006; Nakagomi et al., 1998), and SP-Toyopearl 550C ion exchange column (Motoi & Kodama, 2003).

2.3.3.3 Gel filtration

Gel filtration is an advanced technique to separate peptides. It is commonly applied after ion exchange chromatography, where peptides are separated based on size differences with simultaneous desalting of the samples. Based on the molecular mass ranges of the peptides, columns with various exclusion limits are employed. For example, Sephadex G-10 column (exclusion limit: 700 Da) was used in buckwheat hydrolysates purification (Ma et al., 2006); Sephadex G-15 column (exclusion limit: 1500 Da) was employed in separation of mung bean protein hydrolysates (Li et al., 2006), sea cucumber gelatin hydrolysate (Zhao et al., 2007), and shark meat hydrolysates (Wu et al., 2008). Sephadex G-25 column (exclusion limit: 1500 Da) was applied for the separation of peptides from human plasma hydrolysates (Nakagomi et al., 1998), beef hydrolysates (Jang & Lee, 2005), and douchi (Zhang et al., 2006). Sephadex G-50 column (exclusion limit: 5000 Da) was used to produce active fraction from sunflower hydrolysates (Megías et al., 2004) and fermented oyster sauce (Je et al., 2005). Sephadex LH-20 column (exclusion limit: 4000-5000 Da) was utilized for separation of bioactive

peptides from broccoli hydrolysates (Lee et al., 2006), buckwheat hydrolysates (Ma et al., 2006), and porcine hemoglobin hydrolysate (Yu et al., 2006). Biogel P-2 column (exclusion limit: 1800 Da) was used in wheat gliadin hydrolysate purification process (Motoi & Kodama, 2003).

2.3.3.4 Reverse phase high performance liquid chromatography

Reverse phase high performance liquid chromatography (RP-HPLC) separates peptides by taking advantage of the differences in the hydrophobicities / hydrophilicities of the peptides, as well as differences in their sizes. It is suitable to utilize as the final step of purification because of the high resolution. Generally speaking, each peak in the chromatogram represented one to a few peptide(s), which enables separation and purification of fractions with potent ACE inhibitory activities, prior to their sequencing. RP-HPLC was used to perform as the final step in separation for casein hydrolysates (Contreras et al., 2009; Maeno et al., 1996), human plasma hydrolysates (Nakagomi et al., 1998), fermented oyster sauce (Je et al., 2005), chickpea protein hydrolysates (Pedroche et al., 2002; Yust et al., 2003), buckwheat hydrolysates (Li et al., 2002), sunflower hydrolysates (Megías et al., 2004), beef hydrolysates (Jang & Lee, 2005), mung bean protein hydrolysates (Li et al., 2006), broccoli hydrolysates (Lee et al., 2006), peanut hydrolylsates (Quist et al., 2009), wheat gliadin hydrolysate (Motoi & Kodama, 2003) buckwheat hydrolysates (Ma et al., 2006); porcine hemoglobin hydrolysate (Yu et al., 2006), douchi (Zhang et al., 2006), sea cucumber gelatin hydrolysate (Zhao et al., 2007), and shark meat hydrolysates (Wu et al., 2008).

2.3.4 Sequencing of ACE inhibitory peptides

2.3.4.1 Techniques used for ACE inhibitory peptides sequencing

Two techniques, tandem mass spectrometry (MS/MS) and Edman degradation combined with protein sequencing are extensively applied in bioactive peptides sequencing. Edman degradation is a traditional technique in

which N-terminal amino acids of the peptides are released in sequence and subsequently analyzed by HPLC and identified by their retention times (Aristoy et al., 2008). MS/MS technique is related to fractionation of peptide ions and then analyzing the ions according to different mass-to-charge ratio (m/z) (Sachon & Jensen, 2007). Matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) are two common ways for generating peptide ions, which is then followed by tandem MS, usually a combination of two "time-of-flight mass" analyzers (TOF-TOF), or quadruple analyzers and a "time-of-flight" mass analyzer (Q-TOF) (Sachon & Jensen, 2007).

Both techniques have been extensively used for sequencing of ACE inhibitory peptides. Examples of studies using Edman degradation techniques include those reported by Nakagomi et al. (1998), Li et al. (2002), Motoi & Kodama (2003), Jang & Lee (2005), Lee et al. (2006), Ma et al. (2006), Mallikarjun Goudaa et al. (2006), Qian et al. (2007), and Zhao et al. (2007). Examples of studies using MS/MS techniques are those described by Li et al. (2006), Yu et al. (2006), and Wu et al. (2008).

2.3.4.2 Typical terminal amino acids of ACE inhibitory peptides

Cheung, et al. (1980) have suggested that C-terminal amino acids play a crucial role for peptides delivering ACE inhibitory activities. According to these authors, aromatic amino acids especially tryptophan, was the most favourable C-terminal residue for ACE inhibitory peptides, while tyrosine, phenylalanine and proline were also common. ACE inhibitory peptides were also likely to have branched-chain aliphatic amino acids, such as valine and isoleucine as their N-terminal amino acids. This knowledge was useful for ACE inhibitory peptides synthesis, and is supported by many food protein derived bioactive peptides sequences as well (Table 2-2).

However, several purified peptides with potent ACE inhibitory activity had discrepancies from the profiles described above. These peptides include YPK, a potent ACE inhibitor derived from broccoli with IC_{50} value of 10.5 μg/mL, which

had an aliphatic amino acid in its C-terminal and an aromatic amino acid in its N-terminal (Lee et al., 2006). Other examples include ERKIKVYL from peptic digest of ovalbumin (Fujita et al., 2000), VLAQYK from beef hydrolysate (Jang & Lee, 2005), PNRIKYGD and PTHIKWGD from bonito meat (Hasan et al., 2006) and VVYPWT from pepsin digest of porcine hemoglobin (Yu et al., 2006). So far, typical profiles of ACE inhibitory amino acids remain obscure and require further study.

Table 2-2 Dietary protein derived ACE inhibitory peptides with F/P/W/Y at C-terminal

Peptide sequence	Origin	IC_{50}a (μM)	Reference
IHPF	Milk fermented with *Lactobacillus rhamnosus*	193.9	(Hernández-Ledesma et al., 2004)
LPHF	*Monascus purpureus* acid proteinase digest of soybean	670	(Kuba et al., 2005)
KLPAGTLF	Alcalase digest of mung bean	13.4	(Li et al., 2006)
LGFPTTKTYFPHF	Pepsin digest of porcine hemoglobin	4.92	(Yu et al., 2006)
CF	SM98011 digest of shark meat	1.98	(Wu et al., 2008)
MF	SM98011 digest of shark meat	0.92	(Wu et al., 2008)
LKP	Thermolysin digest of chicken muscle	0.32	(Fujita et al., 2000)
IAP	Pepsin and protease M digest of wheat gliadin	2.7	(Motoi & Kodama, 2003)
DLP	Soy protein alkaline hydrolysate	4.8	(Wu & Ding, 2002)
HLP	Milk fermented with *L. rhamnosus*	210	(Hernández-Ledesma et al., 2004)
LAIPVNKP	*M. purpureus* acid proteinase digest of soybean	70	(Kuba et al., 2005)
VLIVP	Protease P digest of soybean 11S globulin	1.69	(Mallikarjun Gouda et al., 2006)

GPP	Buckwheat	23.1	(Ma et al., 2006)
IKW	Thermolysin digest of chicken muscle	0.21	(Fujita et al., 2000)
LQKW	Thermolysin digest of bovine β-lactoglobulin	34.7	(Hernández-Ledesma et al., 2004)
GTW	Prozyme 6–facilitated lactic acid fermentation of milk whey	464.4	(Chen et al., 2007)
GAW	Prozyme 6–facilitated lactic acid fermentation of milk whey	240.0	(Chen et al., 2007)
FALPQY	Trypsin digest of bovine α_{s2}-casein	4.3	(Tauzin et al., 2002)
YQY	Pepsin, trypsin, chymotrypsin digest of buckwheat	4	(Li et al., 2002)
PSY	Pepsin, trypsin, chymotrypsin digest of buckwheat	16	(Li et al., 2002)
EY	SM98011 digest of shark meat	2.68	(Wu et al., 2008)
RYLGY	Pepsin digest of milk casein	0.71	(Contreras et al., 2009)

[a] IC_{50} = half maximal inhibitory concentration.

2.3.5 ACE inhibitory *in vitro* assays and *in vivo* studies

2.3.5.1 *In vitro* assays

In vitro ACE inhibitory activity study is performed by monitoring the converting reaction by ACE with a particular substrate in the absence/presence of inhibitors (Hernández-Ledesma et al., 2011). Early studies employed angiotensin I as substrate (Skeggs et al., 1956, Yang et al., 1970). However, it is now clear that both the substrates (angiotensin I) and the product (angiotensin II) might be degraded by proteases except ACE found in tissues extracts; and this could lead to confusing results. Accordingly, Cushman & Cheung (1971) developed a novel assay using hippuryl-L-histidyl-L-leucine (HHL) as substrate (Fig. 2-5). This method involved the extraction of hippuric acid (HA), the product released by ACE, by ethyl acetate followed by measurement of absorbance at 228 nm. One unit of

ACE activity could be expressed as producing one µmol of hippuric acid in 1 min at 37 °C. This assay has been further modified by Wu & Ding (2002), who proposed analysis for HA by RP-HPLC. In the modified assay, reactions were stopped by hydrochloric acid and then injected into the RP-HPLC system for analysis. Since retention time of HA and HHL are different, it avoids the extraction step of HA, and improves precision of results.

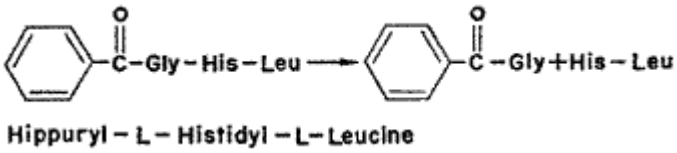


Fig. 2-5. Hydrolysis of HHL by ACE (Modified from Cushman and Cheung, 1971)

Furanacryloyl-phenyl-glycyl-glycine (FA-PGG) was also employed as substrate in ACE inhibitory assays (Holmquist et al., 1979). It involved the reduction in absorbance at 340 nm while FA-PGG was broken down into FA-Phe and Gly-Gly from the action of ACE.

A comparable study was performed between HHL and FA-PGG assays by Shalaby et al. (2006). Whey protein hydrolysate was used as test material. Both assays were recommended by the authors since they gave similar IC_{50} values for the test material**.** However, HHL spectrophotometric assays seem to require more time and labor input (6-12 samples/day) than that of FA-PGG assays (30 samples/day). But it is also recommended to use the HHL spectrophotometric assay if there is interference with the absorption at 340 nm by the extract.

2.3.5.2 *In vivo* studies

Bioactive peptides, when ingested, may experience degradation by various endogenous proteases that may lead to alterations of their bioactivities. Thus, some peptides may show potent ACE inhibitory activities *in vitro*, but may fail to elicit blood pressure lowering effects *in vivo*. An example is the peptide from milk

α_{s1}-casein f(23-27) that exerted no antihypertensive effect at all in animal tests, but displayed a potent *in vitro* IC_{50} (Maruyama et al., 1987). Based on this fact, many studies have been performed on the *in vivo* behaviour of bioactive peptides using model animals. The most commonly used model animal system is spontaneous hypertensive rats (SHR). A broad range of food derived peptides, for example, hydrolysates from milk, sardine muscle, chicken muscle, soy protein, and ovalbumin were administrated to SHR either orally or intravenously, and showed systolic blood pressure responses of +1.3 - -80 mm Hg (Meisel et al., 2005). A few bioactive peptides demonstrated antihypertensive effects of peptides on human volunteers. For example, the tripeptides IPP and VPP, derived from sour milk, were observed to reduce systolic blood pressure (SBP) and diastolic blood pressure (DBP) by -10.1 mm Hg and -9.4 mm Hg, respectively after 8 weeks of treatment; and demonstrated further reductions of -6.7 mm Hg and -3.6 mm Hg for SBP and DBP, respectively after a prolonged 21 weeks of administration (Seppo et al., 2002; Seppo et al., 2003). No side effects were observed after administration of bioactive peptides for antihypertensive subjects, additionally, no significant effects were documented for normotensive subjects (Meisel et al., 2005).

2.3.6 Applications of ACE inhibitory peptides

Meisel et al., (2005) proposed three essential factors for easier consumer acceptance of bioactive peptides/foods, which are efficacy, safety and fine sensory properties. Thus far, a few ACE inhibitory peptides have been successfully commercialized as bioactive supplements introduced to food matrix, such as sour milk with brand name Calpis, produced by Calpis Food Industry co, Ltd, Tokyo, Japan, known to contain IPP (15 mg/L) and VPP (11 mg/L) (Hata et al., 1996). IPP and VPP were also formulated into sour milk Evolus® at concentrations of 15 mg/L and 20 mg/L, respectively, and commercialized by Valio Ltd, Valio, Finland (Seppo et al., 2003). BioZate® produced by DAVISCO foods international, INC, Eden Prairie, USA contains hypotensive whey protein hydrolysates (Pins & Keenan,

2002). As many more fundamental studies are being undertaken, in response to the increasing interests from consumers for products with beneficial health effects, it is almost predictable that the future will be bright for the development of novel foods containing ACE inhibitory peptides.

Connecting Statement to Chapter 3

The importance and the dearth of understanding of the properties of dry bean proteins were described thoroughly in the previous two chapters. Study of dry bean protein properties, such as protein subunit components, thermal properties and structure properties will not only provide new knowledge in this field, but also enable improved understanding for conducting further studies on their potential ACE inhibitory properties. Little information was found in the literature on the characteristics of the major varieties of dry beans grown in Canada. In this chapter, therefore, a comparison study of protein composition, thermal properties, and molecular structure from selected nine dry bean varieties which are largely grown in Canada was undertaken. Protein isolates were produced from the nine varieties of dry beans and were used as materials for all the analysis reported in the thesis. Electrophoresis and reversed-phase high-performance liquid chromatography (RP-HPLC) were employed to study protein composition; differential scanning calorimetry (DSC) was used for the analysis of thermal properties; and Fourier transform infrared spectroscopy (FTIR) was utilized to study the protein secondary structure. The results of this study were presented at the conference shown below and published as an original research peer-reviewed paper in the Food Research International Journal as follows:

Rui, X., Boye, J. I., Ribereau, S., Simpson, B. K., & Prasher, S. O. (2011). Comparative study of the composition and thermal properties of protein isolates prepared from nine *Phaseolus vulgaris* legume varieties, *Food Research International* 44(8):2497-2504.

Rui, X., Boye, J. I., Ribereau, S., Simpson, B. K., & Prasher, S. O. (2010). Comparative study of the molecular structure and thermal properties of protein isolates prepared from nine *Phaseolus Vulgaris* legumes. CIFST/AAFC Conference,

May 30th –June 1st, Winnipeg, MB, Canada, (poster presentation).

Chapter 3: Composition and thermal properties of protein isolates prepared from nine *Phaseolus vulgaris* legume varieties

Abstract

Beans contain high amounts of protein and, as demonstrated with other legumes, may contain peptides with bioactive properties. Dozens of dry bean (*Phaseolus vulgaris*) varieties are grown in Canada, and many vary not only in terms of their protein content but also in their composition. In this study, the composition, molecular structure and thermal properties of nine varieties of *Phaseolus vulgaris* were studied using electrophoresis, reversed-phase high-performance liquid chromatography (RP-HPLC), differential scanning calorimetry (DSC), and Fourier transform infrared (FTIR) spectroscopy. The predominant protein observed in each variety was phaseolin. RP-HPLC chromatogram showed protein isolates (PIs) of navy, great northern and black beans to have different profiles of low-molecular-weight proteins. Additionally, black bean PIs were missing proteins in the molecular mass (MM) range of 60 to 97 kDa. DSC analysis showed cranberry and light red kidney bean PIs to have significantly lower denaturation temperatures compared to the other varieties. RP-HPLC chromatograms of cranberry and light red kidney bean PIs were also very different. FTIR spectroscopy showed the predominant secondary structures in all varieties to be β-sheets and random coils. Increasing the temperature from 25 °C to 95 °C resulted in a reduction in β-sheet structures and an increase in the content of random coils and antiparallel β-sheet structures. Overall there were no major dissimilarities observed in the FTIR profiles of the *nine Phaseolus vulgaris* varieties.

3.1 Introduction

Phaseolus vulgaris is the major bean species cultivated in Canada. Canada's suitable climate and proper soil conditions have led to the country's ranking over the past 10 years as one of the world's top five dry-bean exporters (Food and Agriculture Organization of the United Nations, 1997–2007). Dozens of bean varieties, including navy, pinto, black, dark red kidney, light red kidney, cranberry, small red, great northern, pink, brown, and white kidney, are widely cultivated (Skrypetz, 2000).

The predominant proteins in beans are salt-soluble globulins, including a major fraction of phaseolin and a minor fraction of legumin (Bhatty, 1982; Satterlee et al., 1975). Legumin, with a molecular mass (MM) of 300 to 400 kDa, has six subunits linked by disulfide bonds (Derbyshire et al., 1976). Each subunit can be further divided into an acid subunit with a MM around 40 kDa and a basic subunit with a MM of 20 kDa (Boulter & Croy, 1997). In contrast, phaseolin is a trimer (MM: about 150–190 kDa), with each subunit having a MM of around 50 kDa (Boulter & Croy, 1997; Hall et al., 1977) and with an absence of disulfide bonds between the subunits (Meng & Ma, 2001a; Tang, 2008). Although extensive studies have been done on legumin and phaseolin structures in *Phaseolus vulgaris,* very few studies have reported on specific legumin and phaseolin characteristics such as secondary structures and thermal properties.

Fourier transform infrared (FTIR) spectroscopy is an infrared spectroscopic technique used to analyze the secondary structure of proteins and peptides based on the infrared bands in the amide I region (1700–1600 cm^{-1}) (Surewica & Mantsch, 1988). Secondary structure analyses with FTIR were performed on hyacinth bean (*Dolichos lablab*), rice bean (*Phaseolus calcaratus*), and red bean (*Phaseolus angularis*) (Law et al, 2008; Meng & Ma, 2001b) which suggest a predominance of β–structures.

Thermal properties are another important physicochemical property of interest. This is especially true for legume seeds given that heat treatment is essential to inactivate antinutritional factors, such as trypsin inhibitors and hemagglutinins contained in legumes (Meng & Ma, 2001a). Differential scanning calorimetry (DSC) has proven effective for monitoring thermal transformations during protein denaturation (Biliaderis, 1983). Denaturation temperatures (T_d) for bean proteins reported in the literature range from 84 °C to 91 °C, with kidney and adzuki beans having higher thermal stabilities and T_d values of 90.2 °C and 90.07 °C, respectively (Tang, 2008; Yousif et al., 2003). Thermal properties of pulse proteins affect their functionality. A study on six different Indian chickpea cultivars found a significant ($P \leq 0.05$) negative correlation of T_d with oil absorption capacity (Kaur & Singh, 2007). Furthermore, the authors demonstrated that cultivars with higher enthalpy (ΔH) values had higher water absorption capacity. Additionally, studies on kidney bean found better gelling properties for samples with greater thermal stability (Yin et al., 2010).

Different legume varieties may have dissimilar protein profiles and properties, a phenomenon that could impact bioactivity, functionality and further affect applications in the food industry. Thus, detailed protein characterization studies are required for different legume varieties in order to identify targeted applications in the food industry. Comparison studies have been conducted on protein isolates (PIs) from six varieties of chickpea (*Cicer arietinum* L.) (Kaur & Singh, 2007). However, studies comparing protein profiles, molecular structure and thermal properties of *Phaseolus vulgaris* bean varieties are rare.

In the present study, nine *Phaseolus vulgaris* bean varieties widely grown in Canada were selected for evaluation. Protein isolates were prepared from the seeds and their composition, molecular structure and thermal properties were studied by electrophoresis, mass spectrometry (MS), reversed-phase high-performance liquid chromatography (RP-HPLC), DSC, and FTIR. Furthermore,

possible relationships between molecular structure and thermal properties were considered and are discussed. The results are expected to assist further studies of functional properties as well as bioactive properties for these varieties.

3.2 Materials and methods

3.2.1 Materials

Nine varieties of beans (*Phaseolus vulgaris*), namely navy, pink, pinto, cranberry, black, great northern, dark red kidney, light red kidney, and small red, were selected based on their prominence as major bean types grown in Canada. The beans were provided by Pulse Canada (Winnipeg, MB) and were stored at 4 °C until use.

Low-molecular mass calibration kits were from Amersham Pharmacia Biotech (Uppsala, Sweden). Precast (10-20%) gradient polyacrylamide Tris-HCl gels and Coomassie brilliant blue R-250 were from Bio-Rad Laboratories (Hercules, CA). SEP-PAK C18 cartridges were from Waters (Milford, MA). All other chemical reagents were from Sigma-Aldrich (St. Louis, MO).

3.2.2 Protein isolates (PIs) preparation

The protocol for the preparation of the PIs from the *Phaseolus vulgaris* beans followed an earlier method of Russin et al. (2007) with slight changes. Beans were first ground into a flour with a Retsch/Brinkmann centrifugal mill (Newton, PA) equipped with 0.5-mm ring sieves. Flours were defatted with hexane (1:3, w/v) and air-dried overnight (about 18 h) under a fume hood at room temperature (about 23 °C–25 °C). The defatted flours were first dispersed in 15-fold Millipore water and shaken for 1 h at 55 °C with the pH adjusted to 9. The slurry was then centrifuged at 11,000 *g* at 20 °C for 30 min, and the precipitates were discarded. The collected supernatants were stored at 4 °C overnight (about 15 h) to allow for precipitation of starch and then centrifuged as above. The supernatant obtained was subjected to acid precipitation by

adjusting the pH to 4.5 using 1M HCl solution. After centrifugation using the same conditions as above, the recovered precipitates were washed two more times with Millipore water at 1:5 (w/w). The precipitates obtained were resolubilized in Millipore water at pH 7 and freeze-dried.

3.2.3 Proximate analysis

Proximate analysis was conducted on both the bean flours and the PIs. Moisture analysis was conducted according to the official method of the American Association of Cereal Chemists (AACC) method 44-15A (American Association of Cereal Chemists, 2003) by drying the samples (about 2 g for bean powders and about 0.5 g for bean PIs) in a Fisher Isotemp vacuum oven (Fisher Scientific, Hampton, NH). Ash measurement was conducted according to the AACC method 08-03 (American Association of Cereal Chemists, 2003). Total protein content was determined with the Dumas method, as described in the AOAC method 992.15 (AOAC International, 1995), with a LECO instrument (LECO Corp., St. Joseph, MI). A factor of 6.25 was used for the conversion of nitrogen to protein. For the bean flours, lipid analysis was carried out by using both a Soxhlet extraction (Method 30-25, American Association of Cereal Chemists, 2003) and a LECO instrument (TFE2000, LECO Corp.) using supercritical CO_2 extraction as per Chandrasekar et al. (2001). Due to the limited sample size, lipid contents in the PIs were measured only with a LECO instrument which required less material. Total carbohydrate contents were determined by difference. All sample analyses were performed in triplicate, except for lipid determinations by supercritical CO_2 extraction, which were performed in duplicate.

3.2.4 Electrophoresis

The freeze-dried PIs of the nine bean varieties were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was carried out under both reducing (presence of β-mercaptoethanol [β-ME]) and non-reducing (absence of β-ME) conditions. Pre-cast (10-20%) gradient

polyacrylamide Tris-HCl gels were used with a Bio-Rad Criterion Cell under a constant voltage of 200 V. Low-molecular mass (14.4–97 kDa) calibration kits from Amersham Pharmacia Biotech were used as standard markers. After staining with Coomassie brilliant blue R-250, the gel was scanned with a Bio-Rad GS-690 calibrated imaging densitometer and then analyzed using a Multi-Analyst/PC Analysis software (Bio-Rad).

3.2.5 Reversed-phase high-performance liquid chromatography

RP-HPLC analysis was performed with a Jupiter C4 reversed-phase analytical column (250 mm × 4.6 mm) (Phenomenex, Torrance, CA). The PIs (containing 5 mg protein) were dispersed in trifluoroacetic acid (TFA) solution (1 mL, 0.1%) and centrifuged at 503 g for 3 min. The supernatants were collected, and 50 µL of each sample was injected into the column. The mobile phase comprised two buffers: solvent A (0.1% TFA in Millipore water) and solvent B (0.1% TFA in acetonitrile [ACN]). The time program started at 30% B and then increased to 40% B in 5 min in a linear gradient. The elution was held at 40% B for 5 min and then increased to 50% in 10 min and maintained there for another 5 min. Solvent B was then increased to 100% in 5 min, held for 1 min, and then dropped to 20% in 0.4 min. The flow rate was set at 0.8 mL/min, and the elution was monitored at 280 nm.

3.2.6 Protein in-gel tryptic digestion

Twelve bands from the electrophoresis studies were selected for further analysis by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). Electrophoresis was performed on three varieties (navy, cranberry, and small red beans), and then the gel was rinsed three times for 5 min each time with autoclaved Millipore water followed by staining with Bio-Safe Coomassie for 1 h at room temperature and destaining with Millipore water for a further 1 h with gentle agitation. The target bands were carefully cut with a spot picker (Gelcompany, San Francisco, CA) and transferred to

microcentrifuge tubes that had been prewashed twice with 50% ACN/0.1% TFA. The gel slices were destained twice for 45 min at 37 °C with 1 mL and 0.2 mL 100 mM NH_4HCO_3/50% ACN, respectively. Afterward, 200 µL 100% ACN was added for dehydration with agitation for 5 min. The ACN was later removed with a Zymark TurboVap LV evaporator (Lab Extreme, Kent City, MI). Trypsin digestive solution (1 µg/µL) was prepared by dissolving trypsin in 50 mM acetic acid and the solution was then diluted to 20 µg/mL with 40 mM NH_4HCO_3/10% ACN. To each microcentrifuge tube, 40 µL trypsin digestive solution was added for pre-incubation at room temperature for 1 h, after which 400 µL buffer (40 mM NH4HCO3/10% ACN) was added and incubated overnight at 37 °C. After digestion, 150 µL Millipore water was added and vortexed frequently for 10 min. The liquid was saved, and the gel slices were re-extracted twice with 50 µL 50% ACN/5% TFA at room temperature for 1 h each time. All the extracts were combined and dried in the Zymark TurboVap LV evaporator. The extracts were later desalted by solid-phase extraction on Waters SEP-PAK C18 cartridges and eluted with 10 µL 70% ACN/0.1% TFA.

3.2.7 Liquid chromatography/electrospray ionization tandem mass spectrometry analysis of tryptic bean protein isolate digests

Analysis was performed in a positive ion electrospray mode (+ESI) on a Micromass Waters Q-TOF Ultima Global mass spectrometer equipped with a Z-spray ion source and a NanoLockSpray source (Waters, Mississauga, ON). The analysis on the Q-TOF Global was carried out in V-mode with an instrument resolution between 9000 and 10,000 based on FWHM (full width at half maximum). The source and desolvation temperatures were set to 80 °C and 150 °C, respectively. The Q-TOF was operated at an acceleration voltage of 9.1 kV, a cone voltage of 100 V, an RF (radio frequency) lens voltage of 45 V, and a capillary voltage of 3.8 kV.

Each tryptic digest sample (1–10 µL) was injected onto the C18 trap column coupled to a Waters C18 NanoEase capillary column (75 µm × 50 mm) and was

eluted with one of two buffer systems, ACN or 0.1% TFA in water. The elution was started with 10% ACN and increased to 90% in 75 min in a linear gradient manner. Argon was used as collision gas at 1 bar, and collision energy was set to 10 V for the MS run and 25 to 40 V for MS/MS. Glu-fibrinopeptide from Sigma-Aldrich (Oakville, ON) was used to calibrate the instrument in MS/MS mode. The data were acquired using a data-dependent acquisition (DDA) method, and ProteinLynx software (Micromass, Manchester, UK) was used to automatically transform the MS/MS spectra and interpret the amino acid sequences. All measurements were taken at room temperature. MassLynx 4.0 chromatographic software (Micromass) was used to control the equipment and analyze the data.

3.2.8 Differential scanning calorimetry

The thermal properties of the bean PIs were determined by DSC with a 2910 modulated differential scanning calorimeter (TA Instruments, New Castle, DE) calibrated with indium as standard. The protocol followed a previous study on soybean glycinin (L'Hocine et al., 2007). Each PI was prepared in 0.01 M phosphate buffer (pH 7.4) with a concentration of 10% (w/v). 20 µL of each solution was added to aluminum DSC pans. The pans were hermetically sealed and heated under helium from 20 °C to 120 °C at a rate of 5 °C/min. A sealed pan with 20 µL phosphate buffer was used as a reference. The onset temperature (T_o), peak transition temperature (T_d), width of peak at half-height (ΔT½), and enthalpies (ΔH) were recorded with the Universal Analysis software from TA Instruments. The ΔH values were displayed based on protein weight. All samples were analyzed in triplicate.

3.2.9 Fourier transform infrared spectroscopy

The FTIR analysis was carried out according to an earlier study (L'Hocine et al., 2007). Basically, infrared spectra were recorded at 25 °C with a Bio-Rad FTS 3000 FTIR spectrometer equipped with a deuterated triglycine sulfate detector. The amide I band between 1700 and 1600 cm^{-1} was selected as the region of

interest. The PIs were dispersed (100 mg protein/mL) in the phosphate buffer (pH 7) prepared with D_2O one day before analysis. A 10 µL volume of the sample was then held between two Ca_2F windows with a path length of 25 µm. The number of scans was 256, with a resolution of 2 cm^{-1}. For determination of the effects of temperature on secondary structures, a temperature controller (Dwight Analytical Solutions, Toronto, ON) was used to increase the temperature from 25 °C to 95 °C at 5 °C intervals. The equilibration time was set to 7 min for adjustment of the temperature to the desired level. Win-IR Pro software (version 3.4.2.025) was used for deconvolution using a bandwidth of 13 cm^{-1} and a narrowing factor of 2.5.

3.2.10 Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple comparison tests were used to determine the significant differences between means ($P < 0.05$) using SAS software (version 6.12, SAS Institute, Carey, NC).

3.3 Results and discussion

3.3.1 Proximate analysis

All the bean flours presented high protein contents (22.36%-28.50%, dry basis [d.b.]) and carbohydrate (65.06%–72.11%, d.b.). Ash contents ranged from 4.25% to 5.09%. Fairly low lipid contents (1.16%–1.70%, d.b.) were observed using the Soxhlet extraction, whereas higher values (4.03%-4.99%, d.b.) were obtained by employing the supercritical CO_2 extraction technique. Differences observed between the two techniques are likely due to co-extraction of water and absorption of CO_2 in the oil extracted using the supercritical CO_2 technique (Taylor, et al., 1993). The highest protein content was found in great northern bean (28.50%, d.b.), followed by dark red kidney bean (27.09%, d.b.), while cranberry bean had the lowest (22.36%, d.b.). This sequence was almost

unchanged for the protein contents in the extracted PIs. Great northern bean PI had a protein content of 89.25% (d.b.), which was significantly higher than the other varieties, whereas cranberry bean PI had the lowest (83.96%, d.b.). All the bean PIs had more than three times the protein content of the flours, with reduced contents of ash (2.66%-3.70%, d.b.), carbohydrate (4.49-10.03%, d.b.) and lipid (1.97-3.52%, d.b.).

3.3.2 Electrophoresis

Fig. 3-1 shows the SDS-PAGE profiles of the nine *Phaseolus vulgaris* PIs under both non-reducing (Fig. 3-1a) and reducing (Fig. 3-1b) conditions. The results show large similarities in the SDS-PAGE profiles of the nine varieties. The predominant bands observed under both conditions (Figs. 3-1a and 3-1b) had an estimated MM of around 47 kDa and were barely affected by the presence of β-ME (Fig. 3-1b). The bands were considered phaseolin proteins because they were confirmed to be largely present in bean proteins and devoid of disulfide bonds between subunits (Meng & Ma, 2001a; Tang, 2008). Less prominent bands included some very high-MM bands, which were dissociated after the addition of β-ME and were considered to be legumin. Several bands around 31, 25, 21, and 18 kDa were obtained in all varieties and were considered to belong to phytohemagglutinin (PHA) or globulin based on the MS studies described further below (Figs. 3-1a and 3-1b). Similar results were also obtained with other dry beans (Kohnhorst et al., 1991; Makri & Doxastakis, 2006; Meng & Ma, 2001a; Tang, 2008).

Some minor differences in the electrophoresis profiles were found among the nine varieties. Black bean PIs showed the most unique profiles compared to the other varieties, with intense bands in the high-MM range under reducing conditions (lane 5, Fig. 3-1b). These bands are likely large proteins or proteins aggregates stabilized by forces other than disulfide bonds, such as hydrogen bonds, given that β-ME was unable to break down the structure completely. In contrast, bands with MMs ranging from 60 to 97 kDa were missing. Dissimilar

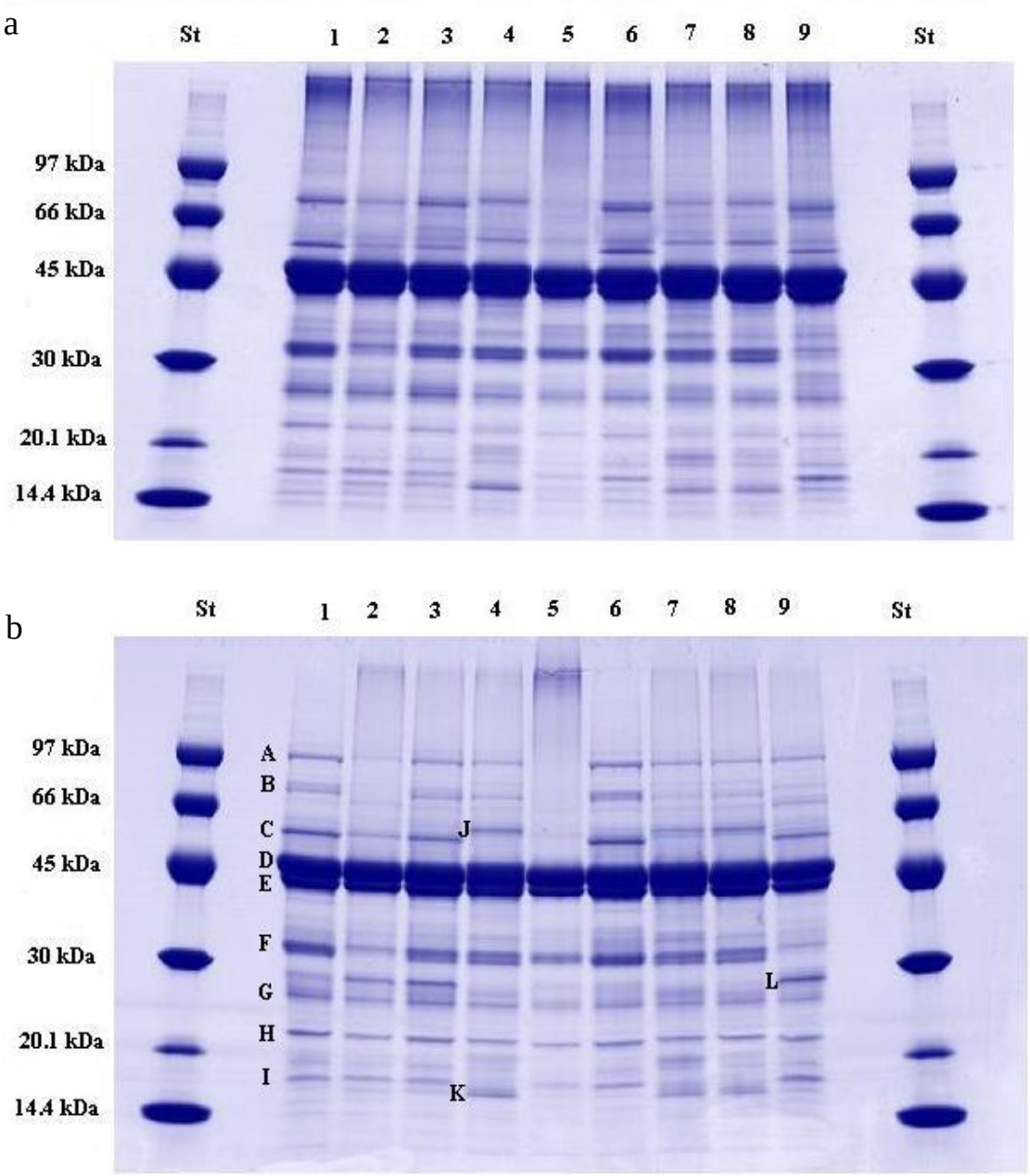


Fig. 3-1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of *Phaseolus vulgaris* bean protein isolates (a) under non-reducing conditions and (b) under reducing conditions (5% β-mercaptoethanol).

(1) Navy bean; (2) pink bean; (3) pinto bean; (4) cranberry bean; (5) black bean; (6) great northern bean; (7) light red kidney bean; (8) dark red kidney bean; and (9) small red bean; A-L epresents the specific band cut-off for liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) analysis.

profiles were observed in the cranberry bean PI (lane 4, Figs. 3-1a and 3-1b), light red kidney bean PI (lane 7, Figs. 3-1a and 3-1b), and the dark red kidney bean PI (lane 8, Figs. 3-1a and 1b), which showed bands with an estimated MM of 63 kDa. The remaining five varieties (navy, pink, pinto, great northern, and small red bean PIs) showed bands with a slightly lower MM of about 60 kDa. Cranberry, light red kidney, and dark red kidney bean PIs also had bands with a MM of 17 kDa, while the other six varieties had bands with a slightly higher MM (18 kDa). Light red kidney bean PI (lane 7, Fig. 3-1a, and 3-1b) showed intense bands at around 35 kDa. Pink bean PI (lane 2, Fig. 3-1b), pinto bean PI (lane 3, Fig. 3-1b), and small red bean PI (lane 9, Fig. 3-1b) had intense bands with a MM of 27 kDa. In addition, extra bands with MMs around 80 and 74 kDa were found in the small red bean PI (lane 9, Fig. 3-1b).

3.3.3 Liquid chromatography/electrospray ionization tandem mass spectrometry analysis of tryptic protein isolate digests

LC/ESI-MS/MS results are shown in Table 3-1. All the bands except A and B were matched to proteins in the database. Bands D and E, the most intense bands observed in the electrophoresis patterns (Fig. 3-1b), were confirmed to be α-type phaseolin and phaseolin (*Phaseolus vulgaris*), respectively. Phaseolin is considered to contain three subunits, α, β, and γ, of which α-type phaseolin has the highest MM (Paaren et al., 1987). Phaseolins were also found in the lower-MM range represented by bands G and H in all varieties. Bands G and H had approximate MMs of 25 and 21 kDa, respectively, and were assigned to β-type and α-type phaseolin, respectively. This is in agreement with an earlier study by Barker et al., (1976) which found that part of the proteins with MM of 23 kDa from *Phaseolus vulgaris* beans had similar characteristics to phaseolin. Band C, which had a MM of 60 kDa and was observed in five PI varieties, namely navy, pink, pinto, great northern, and small red beans, was not identified. Similarly, band J which had a higher MM of 63 kDa, and was found in cranberry, light red kidney, and dark red kidney bean PIs was not identified. These two proteins may

be the same or similar protein with slight changes in composition or degree of glycosylation. The electrophoresis pattern of black bean PI was missing both bands. Although no clear assignments were determined from the MS results, the 60 kDa fraction may likely be a breakdown product of 11S legumin (Derbyshire & Boulter, 1976), as reported later for navy bean (Kohnhorst et al., 1991) and red bean (Meng & Ma, 2001a).

Table 3-1 Identification of *Phaseolus vulgaris* bean proteins by LC/ESI-MS/MS Analysis

Electrophoresis Band [1]	MM (Electrophoresis)	MM (MS)	Sequence	Score
A	99 kDa		ATFLEGIITSLPTLGAGQSAFK	40
B	82 kDa		SLLWLMLANNK	27
C	60 kDa	48.486 kDa	Unnamed protein product (*Phaseolus Vulgaris*)	110
D	47 kDa	49.241 kDa	Phaseolin (α-type)	879
E	44 kDa	47.525 kDa	Phaseolin (*Phaseolus Vulgaris*)	884
F	31 kDa	29.728 kDa	PHA-E	423
		29.538 kDa	PHA-L	231
G	25 kDa	47.536 kDa	Phaseolin (β-type)	463
H	21 kDa	49.241 kDa	Phaseolin (α-type)	137
I	18 kDa	15.395 kDa	α-amylase inhibitor β subunit PHA-I β subunit	234
J	63 kDa	48.486 kDa	Unnamed protein product (*Phaseolus Vulgaris*)	129
K	17 kDa	15.395 kDa	α-amylase inhibitor β subunit PHA-I β subunit	96
L	27 kDa	28.964 kDa	α-amylase inhibitor (*Phaseolus Vulgaris*)	204

[1]The letters (A—L) represent the electrophoresis bands selected from Fig. 3-1b for LC-MS analysis.

Some antinutritional proteins were also identified by mass spectrometry. Band F were assigned to both erythroagglutinating phytohemagglutinin (E-PHA), with a MM of 29.728 kDa, and leucoagglutinating phytohemagglutinin (F-PHA), with a MM of 29.538 kDa. Phytohemagglutinin is largely present in beans, with a MM ranging from 27 to 37 kDa (Bollini & Chrispeels, 1978; Makri & Doxastakis, 2006). Compared to the other varieties, pink and small red beans were found to have low amounts of PHA. Band I (18 kDa), which was observed in six PI varieties (navy, pink, pinto, black, great northern, and small red beans), and band K (17 kDa), which was observed in cranberry, light red kidney, and dark red kidney bean PIs, were both assigned to the β-subunit of α-amylase inhibitor. The slight difference in MM was probably due to different amounts of sugar binding to the peptides, as α-amylase inhibitor exists in different glycoforms (Powers & Whitaker, 1977). Similar bands obtained in pink, pinto, and small red beans PIs with a MM of 27 kDa were also identified as α-amylase inhibitor. These bands were considered to be aggregates of α-amylase inhibitor subunits (Moreno et al., 1990).

3.3.4 Reversed-phase high-performance liquid chromatography

Fig. 3-2 shows the HPLC chromatograms of the nine *Phaseolus vulgaris* PIs. Three major fractions (designated as fractions A, B, and C) were observed for most of the bean varieties having retention times of 9.7, 17.7, and 30.9 min, respectively. A reduced amount of fraction A was found in navy and great northern bean PIs, while two split peaks were observed in the black bean PI. Early-eluting fractions with higher hydrophilicity were considered to be components with very low MM (≤22 kDa), as suggested by previous studies on soybean proteins (Peterson & Wolf, 1988). Thus, those three bean PIs were presumed to have different profiles of proteins in the low-MM range. Fraction B seemed to consist of two fractions with very close retention times. Two subfractions were clearly present in the chromatograms of cranberry and light red kidney bean PIs, which were different from those of the other seven varieties

(lanes 4 and 7, Fig. 3-2). This fraction was considered to be phaseolin, with the earlier-eluting sub-fraction assigned to more ionic glycosylated α-type subunits and the later subfraction composed of less ionic glycosylated β- and γ-type subunits (Paaren et al., 1987). The different profiles found in cranberry and light red kidney bean PIs might be explained by their dissimilar profiles in phaseolin. Brown et al., (1981) categorized phaseolin subunits into 'S', 'T', and 'C' categories after the cultivars Sanilac, Tendergreen and Contender, respectively. Deshpande & Nielsen (1987b) found that the phaseolin of cranberry, light red kidney and dark red kidney bean belong to a 'T' type, while the other investigated varieties such as pink and great northern beans have phaseolins belonging to the 'S' type. Interestingly, although dark red kidney phaseolin is classified as a 'T' type phaseolin, its RP-HPLC chromatogram is dissimilar to that of cranberry and light red kidney bean. Fraction C, considered to be more hydrophobic, may be undissociated proteins present in the isolates, based on earlier RP-HPLC results reported for white kidney beans (Alli et al., 1993).

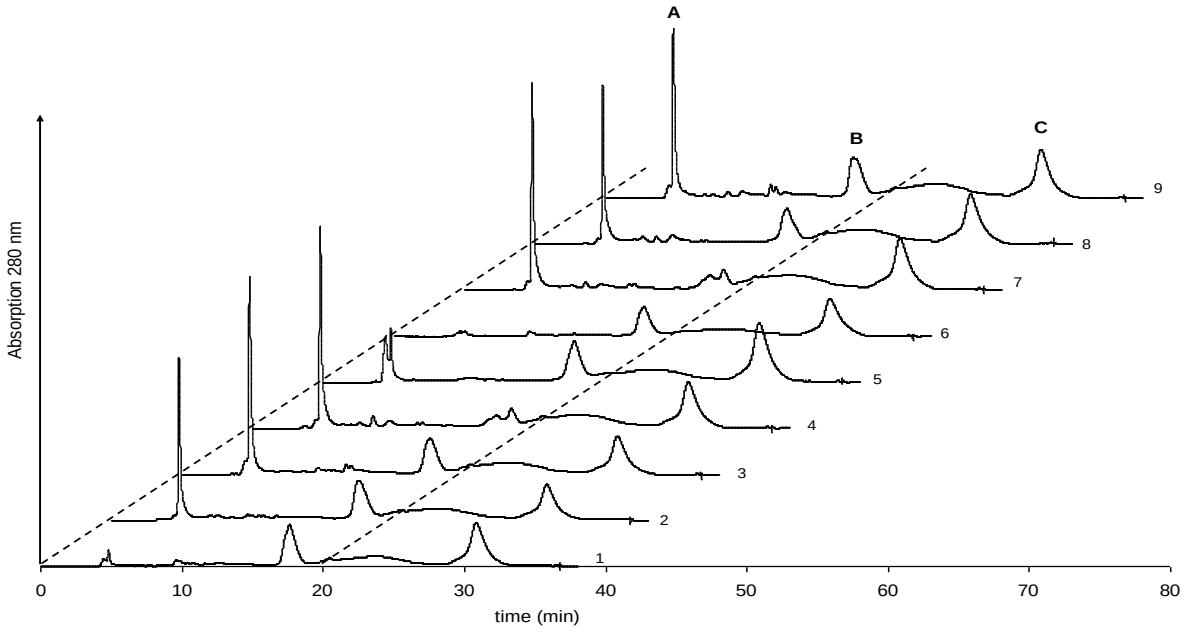


Fig. 3-2. Reversed-phase high-performance liquid chromatography (RP-HPLC) patterns of *Phaseolus vulgaris* bean protein isolates.
(1) navy bean; (2) pink bean; (3) pinto bean; (4) cranberry bean; (5) black bean; (6) great northern bean; (7) light red kidney bean; (8) dark red kidney bean; and (9) small red bean.

3.3.5 Differential scanning calorimetry

The DSC thermograms of all bean PIs showed a single endothermic peak, which likely represents the denaturation of 7S phaseolin. T_d values for navy (90.58 °C), pinto (91.04 °C), dark red kidney (91.14 °C), and small red (90.81 °C) bean PIs were significantly higher than the T_d values for the other varieties (Table 3-2), while cranberry and light red kidney bean PIs had lower Td values, namely 84.99 °C and 82.14 °C, respectively. As T_d values are indicative of the thermal stability of globular proteins (Tang, 2008), the results suggest that cranberry and light red kidney bean PIs may have significantly lower thermal stabilities compared to the other varieties. These differences might be attributable to their classification as 'T' type phaseolins as shown in the RP-HPLC chromatograms. Light red kidney bean PIs, on the other hand, showed significantly lower denaturation ΔH values compared to the other varieties (Table 3-2), representing a lower ordered secondary structure content (Koshiyama et al., 1981). Lower cooperativity of the protein denaturation transition (ΔT½) was also found in cranberry bean PI (Table 3-2).

In some other DSC studies of bean PIs, including studies on red bean (*Phaseolus angularis*) globulin (Meng & Ma, 2001a) and *Dolichos lablab* vicilin (Law et al., 2008), two endothermic peaks were obtained in the thermograms. This phenomenon was explained as the independent transitions in different subunits of the trimeric structure of vicilin (Law et al., 2008). In the present study, however, only one endothermic peak was obtained for all varieties, suggesting that the three subunits were denaturing codependently (Harwalkar & Ma, 1987). Similar observations were made with *P. calcaratus* vicilin (Law et al., 2008), red kidney bean (*Phaseolus vulgaris*) PIs, and mung bean (*Phaseolus aureus*) PIs (Tang, 2008).

Compared to T_d values for other bean species, including red bean globulin (86.4 °C) (Meng & Ma, 2001a), red kidney bean (90.2 °C), mung bean (*Phaseolus aureus*) (84.6 °C) (Law et al., 2008), *Dolichos lablab* vicilin (90.9 °C), and

P. calcaratus vicilin (91.9 °C) (Tang, 2008), the T_d values for most of the varieties in the present investigation were higher (88.97 °C–91.14 °C), with the exception of two varieties, namely cranberry and light red kidney bean PIs.

Table 3-2 DSC characteristics of *Phaseolus vulgaris* bean protein isolates [1,2]

	T_o [3] (°C)	T_d [4] (°C)	ΔH [5] (J/g)	$\Delta T_{½}$ [6] (°C)
Navy	85.33 (0.13)^{b}	90.58 (0.50)^{a}	10.98 (0.04)^{bc}	4.98 (0.04)^{d}
Pink	84.70 (0.06)^{c}	89.55 (0.06)^{b}	12.14 (0.53)^{a}	5.15 (0.13)^{cd}
Pinto	85.73 (0.08)^{ab}	91.04 (0.38)^{a}	10.42 (0.43)^{c}	5.07 (0.10)^{cd}
Cranberry	78.42 (0.41)^{e}	84.99 (0.19)^{d}	10.41 (0.16)^{c}	7.43 (0.06)^{a}
Black	84.18 (0.10)^{d}	88.97 (0.21)^{c}	10.43 (0.59)^{c}	5.17 (0.15)^{c}
Great Northern	84.41 (0.22)^{cd}	89.26 (0.54)^{bc}	11.09 (0.27)^{bc}	5.19 (0.11)^{c}
Light Red Kidney	77.13 (0.44)^{f}	82.14 (0.35)^{e}	6.72 (0.52)^{d}	5.98 (0.14)^{b}
Dark Red Kidney	85.77 (0.10)^{a}	91.14 (0.06)^{a}	11.06 (0.69)^{bc}	4.97 (0.03)^{d}
Small Red	85.46 (0.10)^{ab}	90.81 (0.13)^{a}	11.78 (0.08)^{ab}	5.03 (0.09)^{cd}

[1] Values in brackets represent the standard deviations.

[2 a-f] Values followed by different letters in the same column are significantly different ($P<0.05$).

[3] T_o: Onset temperature.

[4] T_d: Peak denaturation temperature.

[5] ΔH: Enthalpy of denaturation.

[6] $\Delta T_{½}$: Peak width at half-height.

3.3.6 Fourier transform infrared spectroscopy

The FTIR spectra (1700–1600 cm^{-1}) of the nine varieties of *Phaseolus vulgaris* bean PIs are shown in Fig. 3-3. Several major bands were observed in this region. The assignments of these bands were in accordance with previous studies (Table 3-3) (Byler & Susi, 1986; Surewicz & Mantsch, 1988). For all the varieties, the predominant bands observed were β-sheets (1637 cm^{-1}) and random coils (1646 cm^{-1}). These results are consistent with those of earlier studies on other legume proteins, which found them to contain large quantities of β-sheet and random coil structures (Law et al., 2008; L'Hocine et al., 2007; Meng & Ma, 2001b). Plant globulins were also found by Marcone et al., (1998) to have very low α-helix contents.

Only a few minor dissimilarities were observed in the secondary structure profiles of the nine *Phaseolus vulgaris* varieties. For example, higher levels of α-helix structures (1653 cm^{-1}) were observed in light red kidney bean PIs (lane 7, Fig. 3-3), while lower levels of β-strand structures (1627 cm^{-1}) were obtained in cranberry bean PIs (lane 4, Fig. 3-3).

The effects of heat treatment on the secondary structures of the nine *Phaseolus vulgaris* PIs were also investigated. Four varieties representing typical profiles are presented in Fig. 3-4. For all nine varieties, increasing the temperature from 25 °C to 95 °C increased the level of random coil bands (1646 cm^{-1}) and consistently decreased the β-sheet structure content (1637 cm^{-1}) (Fig. 3-4). The shapes of the spectra changed gradually at every temperature level. However, two stages of conformation transformation were observed in the spectra. For all varieties, the first transformation took place around 50 °C to 60 °C, where the β-sheet structure was extensively reduced while large levels of random coil structures could be observed (Fig. 3-4). These changes indicate the initial phase of protein denaturation. Previous studies reported a similar transition temperature range (60 °C–65 °C) and attributed this transformation to protein denaturation (Meng & Ma, 2001b; Nagano et al., 1995). The second

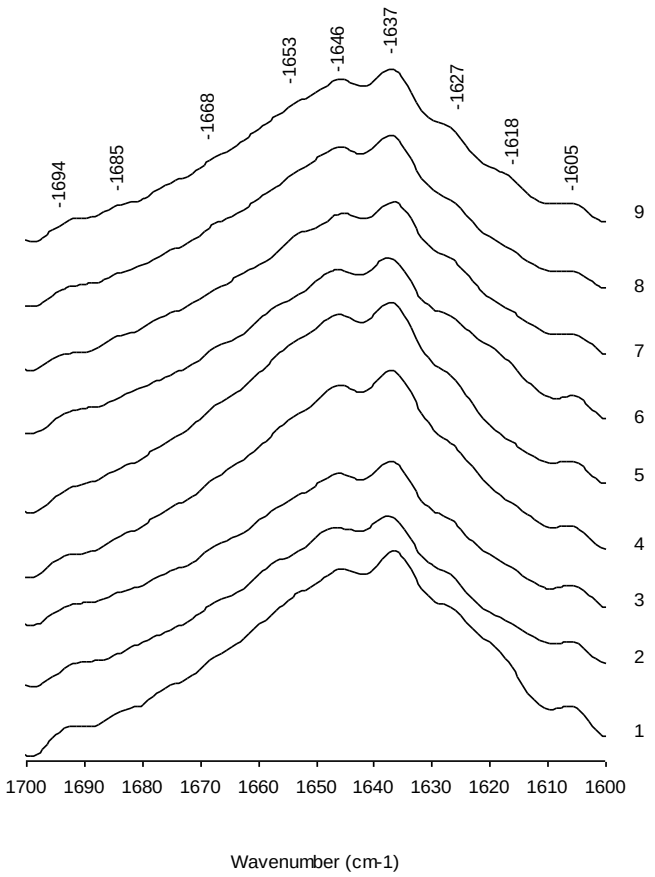


Fig. 3-3. Stacked plot of the deconvolved infrared spectra of *Phaseolus vulgaris* bean protein isolates recorded at 25 °C.

(1) navy bean; (2) pink bean; (3) pinto bean; (4) cranberry bean; (5) black bean; (6) great northern bean; (7) light red kidney bean; (8) dark red kidney bean; and (9) small red bean.

Table 3- 3 Assignment of deconvolved FTIR bands

Wavenumber (cm^{-1})	Assignment
1605	Side-chain vibrations
1618	Anti-parallel β-sheet (intermolecular)
1627	β-strand
1637	β-sheet (intramolecular)
1646	Random coil
1653	α-helix
1668	β-turns
1685	Anti-parallel β-sheet (intermolecular)
1694	β-type

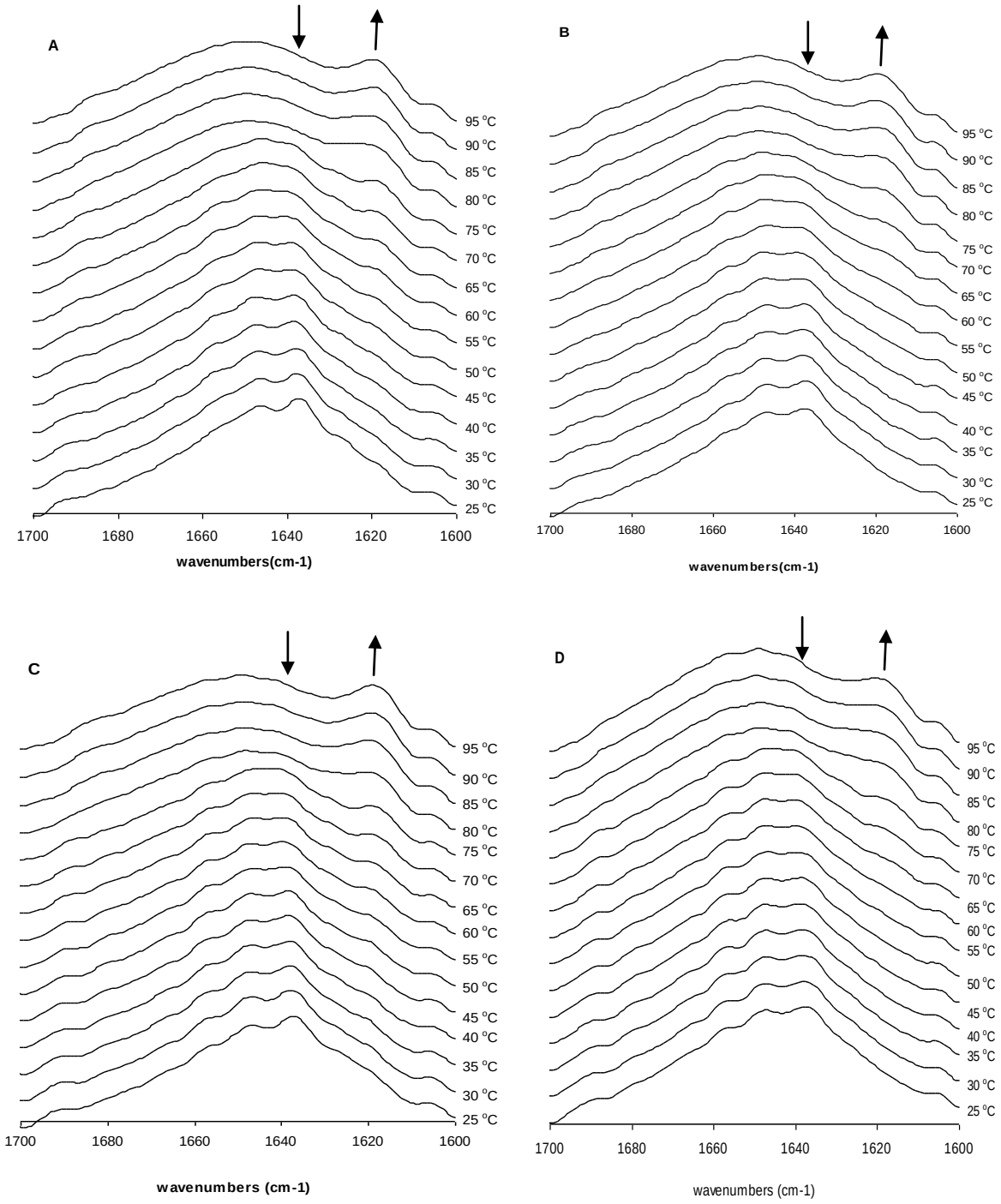


Fig. 3-4. Stacked plot of the deconvolved infrared spectra of representative *Phaseolus vulgaris* bean protein isolates heated from 25 °C to 95 °C.
(A) navy bean; (B) cranberry bean; (C) great northern bean; and (D) dark red kidney bean.
Down and up arrows represented the band intensity at the corresponding spectrum became lower and higher, respectively with the rise of temperature.

transformation occurred at around 80 °C, where intense bands were observed at 1620 cm^{-1}, indicating an increase in intermolecular β-sheet structures as a result of protein thermal aggregation. For some varieties, the formation of intermolecular β-sheet structures occurred at lower temperatures, such as 60 °C to 65 °C for navy and great northern bean PIs (Figs. 3-4A and 3-4C). Pink and small red bean PIs presented similar profiles (data not shown). In contrast, some of the other varieties, such as cranberry and dark red kidney bean PIs, showed relatively higher temperatures for the formation of intermolecular β-sheet structures (70 – 75 °C, Figs. 3-4B and 3-4D). Similar results were obtained for pink, black, and light red kidney bean PIs (data not shown). Based on these results, it appears that protein denaturation and thermal aggregation occurs either at the same time or at different times depending on the bean variety.

3.4 Conclusions

The present investigation showed that the nine varieties of *Phaseolus vulgaris* PIs had differences in protein composition, molecular structure and thermal stability. Two varieties, namely, Cranberry and light red kidney bean PIs were found to have dissimilar properties when compared to the other seven varieties by having significantly lower denaturation temperatures as well as different electrophoresis and RP-HPLC chromatograms. Also, Black bean PIs were missing some proteins in the MM range of 60-97 kDa and had larger amounts of high MM proteins, and navy, great northern, and black bean PIs also had different profiles especially in the low-MM region. As protein function, both biological as well as physicochemical, is influenced by protein composition, the results show the importance of selecting the right bean cultivar for different applications. Further studies are ongoing in our lab to assess the impact of these differences on the bioactive properties of the nine bean varieties.

Connecting Statement to Chapter 4

A comparative study of the composition, thermal properties, and molecular structure of protein isolates extracted from nine varieties of dry beans were presented in the previous chapter. Although the investigated varieties shared some common characteristics, distinctive properties were observed for some varieties. For example, cranberry and light red kidney bean protein isolates demonstrated different subunit composition and thermal properties. Additionally, different molecular composition was obtained for black bean protein isolates. Therefore, in the present chapter, investigations were carried out to study how differences in the protein profiles of the dry bean varieties affect their *in vitro* ACE inhibitory activities. The chapter presents results of *in vitro* ACE inhibitory activities of digests for all nine varieties of dry beans produced by either trypsin digestion or *in vitro* gastrointestinal digestion. Digestibility of the dry bean proteins was also investigated. The results of this study have been presented at the conferences indicated below and being prepared as a paper to be submitted to Journal of Nutrition & Food Sciences as shown below:

Rui, X., Boye, J. I., Barbana, C., Simpson, B. K., & Prasher, S. O. (2012) Angiotensin I-converting enzyme inhibitory properties and digestibility of nine varieties of *Phaseolus vulgaris* protein hydrolysates, *Journal of Nutrition & Food Sciences,* 2 (8):156.

Rui, X., Boye, J. I., Barbana, C., Simpson, B. K., & Prasher, S. O. (2010). Angiotensin I-converting enzyme *in vitro* inhibitory activity of nine *Phaseolus vulgaris* legume tryptic hydrolysates. 8th Canadian Pulse Research Workshop, November 3rd – 5th, Calgary, AB, Canada (poster presentation).

Rui, X., Boye, J. I., Barbana, C., Simpson, B. K., & Prasher, S. O. (2012). Digestibility

and angiotensin I-converting enzyme inhibitory properties of nine varieties *of Phaseolus vulgaris* protein hydrolysates. IFT 2012 Annual Meeting, June 25th–28th, Las Vegas, NV, USA (poster presentation).

Chapter 4: Digestibility and angiotensin I-converting enzyme inhibitory properties of nine varieties of *Phaseolus vulgaris* protein hydrolysates

Abstract

Nine dry bean (*Phaseolus vulgaris*) varieties largely grown in Canada were subjected to digestion using trypsin and *in vitro* gastrointestinal simulation (GIS) followed by a study of their *in vitro* ACE inhibitory properties and digestibility. GIS hydrolysates of all varieties presented significantly higher ACE inhibitory activities and degree of hydrolysis (DH) compared to those of trypsin hydrolysates ($P<0.05$). Cranberry and light red kidney bean protein isolates contained 'T' type phaseolin and had higher DH values during both digestions, with average ACE inhibitory activities (281.7–281.8 µg protein/mL and 141.6–185.1 µg protein/mL respectively for tryptic and GIS hydrolysates) The other seven bean varieties contained 'S' type phaseolin, and of these small red bean showed the lowest ACE inhibitory activities for both trypsin (IC_{50} of 170 µg protein/mL) and GIS digestion (IC_{50} of 118 µg protein/mL), followed by navy bean, with IC_{50} of 200 µg protein/mL (trypsin digestion) and 137 µg protein/mL (GIS digestion). The results demonstrated that both methods of digestions yielded bioactive peptides, however, differing peptide profiles of the bean protein hydrolysates affected their *in vitro* ACE inhibitory properties.

4.1 Introduction

Peptides with hypotensive properties have received increasing attention in recent times. Hypertension is ranked as one of the world's most common high-risk diseases, affecting about 22% of the world's population (International Society of Hypertension, 2010). Angiotensin I-converting enzyme (ACE, EC 3.4.15.1) is the key enzyme responsible for elevation of blood pressure as it is

capable of converting the decapeptide, angiotensin I, to the octapeptide, angiotensin II, a potent vasoconstrictor, and also degrading bradykinin, a vasodilator, into inactive peptides (Yang et al., 1970). Adverse effects resulting from the use of synthetic ACE inhibitors, has enhanced research activities which aimed at investigating and identifying natural sources of ACE inhibitory peptides, because they are considered to be freer of side effects.

Pulses, including pea, lentil, chickpea, and beans contain high levels of protein, fiber, as well as valuable minerals and vitamins which enhance their health-benefitting attributes. ACE inhibitory studies have been conducted on a few pulses, including chickpea (Barbana & Boye, 2010; Pedroche et al., 2002; Yust et al., 2003), pea (Barbana & Boye, 2010; Vermeirssen et al., 2004; Vermeirssen et al., 2005), and lentil (Boye et al., 2010). However, very few studies have investigated the antihypertensive properties of beans (Akıllıoğlu & Karakaya, 2009; Torruco-Uco et al., 2009). An essential step for the generation of bioactive peptides from foods is enzymatic hydrolysis. Bean proteins have lower digestibility compared to animal proteins which can limit their nutritional value (Deshpande & Nielsen, 1987b). In the current study, the digestibility of proteins from nine varieties of dry beans (*Phaseolus vulgaris*) that are largely cultivated in Canada, namely navy, pink, pinto, cranberry, black, great northern, light red kidney, dark red kidney, and small red bean, were studied using trypsin and *in vitro* gastrointestinal digestion simulation (GIS). Subsequently, the *in vitro* ACE inhibitory properties of the protein digests were analyzed and compared to determine the bean variety with highest potential for ACE inhibition under the conditions studied.

4.2 Materials and methods

4.2.1 Materials

Protein isolates of nine varieties of beans (*Phaseolus vulgaris*), namely navy, pink, pinto, cranberry, black, great northern, dark red kidney, light red kidney, and small red beans, were prepared as described in Rui et al. (2011). Low molecular weight calibration kits were from Amersham Pharmacia Biotech (Uppsala, Sweden). Precast 10-20% gradient tris/tricine gels, precast 16.5% tris/tricine gels, tricine sample buffer, and coomassie brilliant blue G 250 were from Bio-Rad Laboratories (Hercules, CA). α-amylase (A 6380), pepsin (P 6887), trypsin (T 0303), α-chymotrypsin (C 4129), ACE regent (A 6778), Hippuryl-His-Leu (HHL) (H 1635) and 2,4,6-trinitrobenzenesulphonic acid (TNBS) (P 2297) were purchased from Sigma-Aldrich Co. (Oakville, ON). All other reagents used were of analytical grade.

4.2.2 Protein analysis

Protein/peptide contents in protein hydrolysates were determined with the Dumas combustion method using a LECO FP-428 apparatus (LECO Corp., St. Joseph, MI, USA) (AOAC International, 1995). A factor of 6.25 was used for the conversion of nitrogen to protein.

4.2.3 *In vitro* protein digestibility

In vitro protein digestibility of bean protein isolates were determined according to a previously published method based on measuring pH drop after 10 min of *in vitro* digestion (Hsu et al. 1977).

4.2.4 Protein hydrolysis

Protein hydrolyses using trypsin and *in vitro* gastrointestinal simulation (GIS) digestions were carried out separately in duplicate. Digestions were conducted using a pH-stat apparatus (TIM 865, Radiometer Analytical SAS, Villeurbanne,

France) with substrate (concentration of 2.5% w/v, based on protein content) dispersed in phosphate buffer (0.01 M, pH 8.0) (Trypsin hydrolysis) or glycine buffer (0.01 M, pH 7.0) (GIS hydrolysis). Trypsin digestions were performed with an enzyme to substrate ratio (E/S) of 1:25 (w/w, based on protein content) for 2 h at 37 °C. GIS digestions were started by pre-treatment of the bean protein isolates with α-amylase solution (1 mg/mL, 0.01 M glycine buffer, pH 7.0) at a ratio at 1:12.5 (v/w) at 37 °C for 3 min and then followed by sequential digestions of pepsin, trypsin and α-chymotrypsin with E/S: 1⁄250 (w/w, based on protein content) as described previously in Barbana & Boye (2010). For both trypsin and GIS digestion, enzyme inactivation after sampling was achieved by heating in boiling water for 10 min followed by centrifugation at 12,000 *g*, 4 °C for 20 min. The supernatants were collected and freeze dried for further electrophoresis and ACE inhibition studies. To follow the time course of hydrolysis, aliquots of the hydrolysates were withdrawn at different time intervals during digestion for electrophoretic analysis. The samples were diluted 10 fold with laemmli buffer, heated in boiling water for 10 min and stored at -20 °C until analysis.

4.2.5 Degree of hydrolysis

Degree of hydrolysis (DH, %) was determined based on the reaction of free amino groups with TNBS (Adler-Nissen, 1979). Total number of amino acid groups was determined by hydrolyzing the samples in 6 M HCl at 110 °C for 24 h. A series concentration of L-Leucine was used to generate the standard curve.

4.2.6 *In vitro* angiotensin I-converting enzyme inhibitory activity determination

ACE inhibitory activity assay was performed using the protocol described previously by Wu and Ding (2002), and Barbana and Boye (2010) by monitoring the formation of hippuric acid (HA) and employing Hippuryl-His-Leu (HHL) as substrate using reverse-phase high-performance liquid chromatography (RP-HPLC). Briefly, 50 µL of 2.17 mM HHL, 10 µL of ACE (1.55 mU) and 10 µL of bean

protein hydrolysates were prepared using borate buffer (100 mM, containing 300 mM sodium chloride, pH 8.3) and vortex to initiate the reaction. The reaction was conducted at 37 °C for 30 min with agitated incubation. After terminating the reaction by addition of 85 µL of 1 M HCl, 10 µL of the solution was injected into a 4.60 x 250 mm Aqua C18 reverse-phase column (5 µm particle size, 125 Å, Phenomenex). Samples were eluted using 50% (v/v) methanol in water with 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.5 mL/min for 15 min. The elution was monitored at 228 nm. Absorbance of the HA peak was measured. Blank samples were prepared without addition of enzyme, and control samples were prepared without the addition of bean protein hydrolysates.

ACE inhibitory activity was calculated using the following equation:

ACE inhibitory activity (%) = [(Ac-As)/(Ac-Ab)] x 100

Where Ac is the absorbance of the control, Ab is the absorbance of the blank, and As is the absorbance of the test sample. The IC_{50} values (half maximal inhibitory concentration) were determined by graphed ACE inhibition percentages versus semi-logarithmic values of sample concentrations following an earlier study (Shalaby et al., 2006).

4.2.7 Electrophoresis

SDS-PAGE analyses of the bean trypsin hydrolysates were carried out using a Bio-Rad Criterion Cell (Bio-Rad Laboratories, Inc., Mississauga, ON) with 16.5% tris-tricine gels. GIS hydrolysates were analyzed using 10-20% gradient tris-HCl gels. For studies under denaturing conditions, 5% (v/v) β mercaptoethanol (β Me) was added to the electrophoresis reagents during sample preparation prior to sample loading. Electrophoreses were conducted under constant voltage of 100 V, using Bio-Rad polypeptide standards (1.423–26.625 kDa) and low-molecular mass standard markers (14.4–97 kDa) calibration kits from Amersham Pharmacia Biotech for 16.5% tris-tricine gels and 10 20% gradient tris-HCl gels, respectively. Gels were scanned with a Bio-Rad GS-690 calibrated imaging densitometer and

analyzed using a Multi-Analyst/PC Analysis software (Bio-Rad Laboratories, Inc., Mississauga, ON).

4.2.8 Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple comparison tests were used to determine the significant differences between means ($P < 0.05$) using SAS Server Interface (version 2.0.3, SAS Institute, Cary, NC).

4.3 Results

4.3.1 *In vitro* protein digestibility and degree of hydrolysis

In vitro protein digestibility of the protein isolates extracted from the nine bean varieties ranged from 83.67% (black bean) to 88.19% (great northern bean). The highest values were found for navy bean and great northern bean, at 88.01% and 88.19%, respectively ($P<0.05$) (Table 4-1). Trypsin digestion of the bean protein isolates gave low degree of hydrolysis (DH) values, ranging from 1.72% (dark red kidney bean) to 4.59% (cranberry bean), whereas higher DH values were obtained after *in vitro* GIS digestion, ranging from 7.22% (dark red kidney bean) to 16.12% (pinto bean) (Table 4-1). Among the nine varieties, hydrolysates of cranberry, light red kidney and pinto bean for both methods of digestion exhibited significantly higher DH values than the other six varieties ($P<0.05$).

4.3.2 Electrophoresis

Under both reducing and non-reducing conditions, SDS-PAGE profiles of the trypsin hydrolysates of all investigated varieties showed predominant bands with estimated molecular mass (MM) of 24 kDa (Fig. 4-1a and b). The intensity of the 24 kDa band was much less for the hydrolysates of cranberry and light red kidney, but there were more intense bands at lower MM between 3–6 kDa, and also additional bands around 16 kDa (lane 4 and 7, Fig. 4-1a and b). The hydrolysate of black bean had a large amount of a high MM fraction which remained at the

Table 4-1 *In vitro* digestibility (%) of bean protein isolates and degree of hydrolysis (DH, %) of bean proteins hydrolysed using trypsin and *in vitro* gastrointestinal simulation (GIS) digestion.

	Navy	Pink	Pinto	Cranberry	Black	Great northern	Light red kidney	Dark red kidney	Small red
In vitro digestibility	88.01±0.13^{a}	84.21±0.90^{c}	85.84±0.13^{b}	85.03±1.02^{bc}	83.67±0.64^{c}	88.19±0.64^{a}	84.39±0.13^{bc}	84.30±0.51^{c}	84.21±0.64^{c}
Trypsin DH	3.29±0.03^{d}	2.72±0.36^{f}	3.97±0.20^{c}	4.59±0.02^{a}	1.91±0.04^{g}	2.64±0.03^{f}	4.32±0.04^{b}	1.72±0.05^{g}	3.03±0.08^{e}
GIS DH	8.25±0.83^{cd}	11.92±0.18^{b}	16.12±1.73^{a}	15.16±0.22^{a}	10.28±0.51^{bc}	11.12±2.78^{b}	15.27±0.15^{a}	7.22±0.06^{d}	9.84±0.08^{bc}

In vitro digestibility data was measured in duplicate. Degree of hydrolysis data was measured in triplicate. Values after ± show the standard deviations.

$^{a-g}$ Values followed by different letters in the same row are significantly different ($P<0.05$).

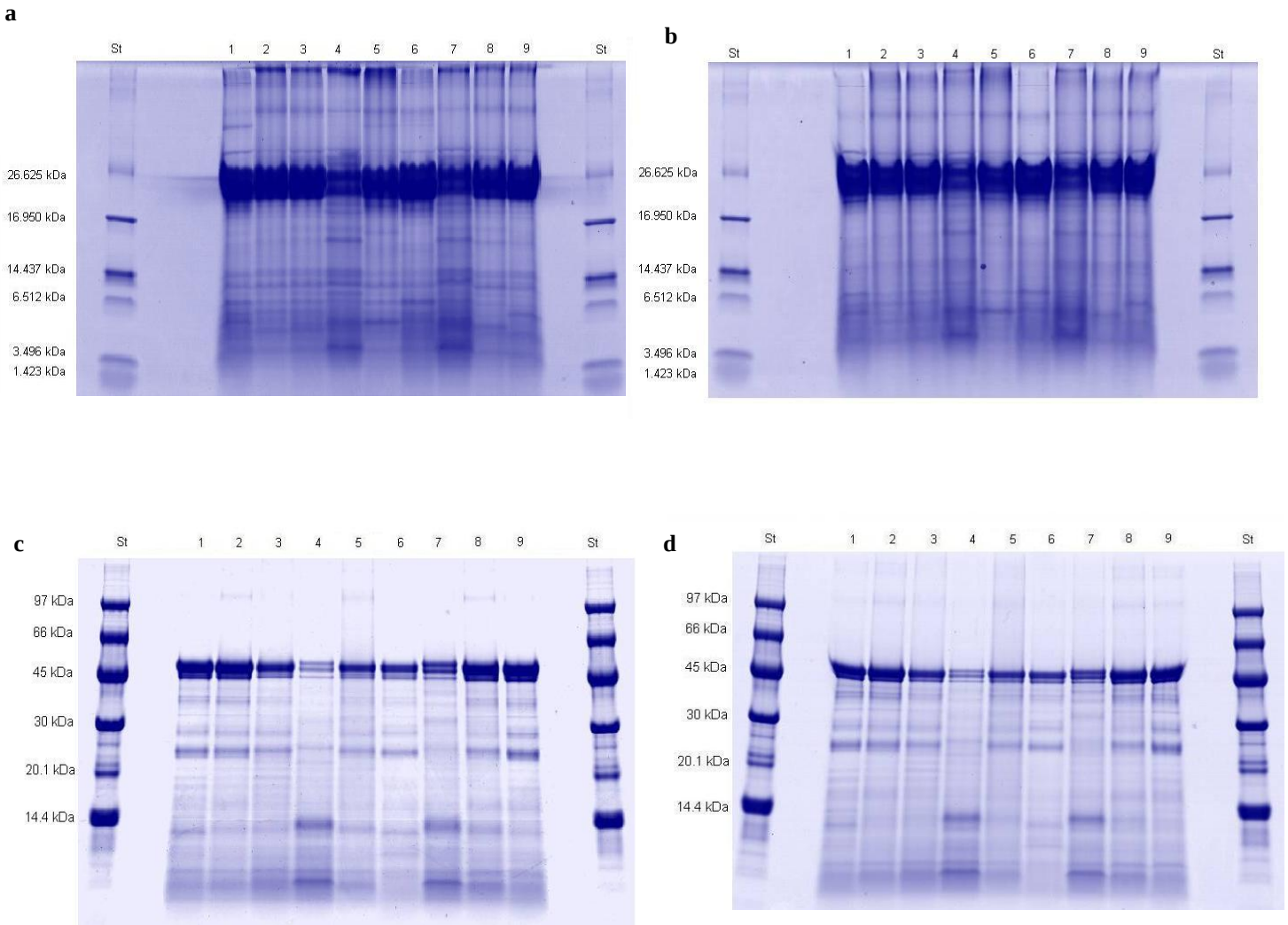


Fig. 4-1. SDS-PAGE of bean protein trypsin hydrolysates (a, b) and *in vitro* gastrointestinal simulation (GIS) hydrolysates (c, d): (a, c) non-reducing conditions; (b, d) reducing conditions (5% β-ME).

Lane 1-9 are 1- navy; 2- pink; 3- pinto; 4- cranberry; 5- black; 6- great northern; 7- light red kidney; 8- dark red kidney; 9- small red.

top of the gel (lane 5, Fig. 4-1a and b); this band was absent for navy and great northern bean hydrolysates (lane 1 and 6, Fig. 4-1a and b).

Unlike the dry bean trypsin digests, the majority of the bands observed in the SDS-PAGE after *in vitro* GIS digestions had a MM around 47 kDa (Fig. 4-1c and d), and no protein/protein aggregates were observed at the top of the gel. Additionally, there was not much of a difference between the samples treated with and without β-ME. Again, cranberry and light red kidney bean hydrolysates showed different profiles compared to the seven other varieties (lane 4 and 7, Fig. 4-1c and d), with the clear evidence of three subunits having MMs of 47, 50, and 53 kDa, whereas the other seven varieties presented only two subunits profiles with MMs of 46 and 50 kDa. Cranberry and light red kidney bean hydrolysates also contained smaller proteins having MMs less than 14 kDa compared to other varieties, and there were fewer bands between the 24 to 38 kDa ranges.

4.3.3 ACE inhibitory activities

All investigated bean varieties showed ACE inhibitory activities after trypsin and *in vitro* GIS digestions. The latter method of hydrolysis yielded significantly lower IC_{50} values compared to the former ($P<0.05$) (Fig. 4-2). Lower IC_{50} values represent higher ACE inhibitory property. Thus, small red bean hydrolysates from both trypsin and GIS digestions, had significantly higher ($P<0.05$) ACE inhibitory activities compared to other varieties undergoing similar digestion, with IC_{50} values of 170 µg protein/mL and 118 µg protein/mL, respectively. Navy bean hydrolysates had the next highest ACE inhibitory activity with IC_{50} values of 200 µg protein/mL (trypsin digestion) and 137 µg protein/mL (GIS digestion). Varieties presenting the least ACE inhibitory activities for both digestions were black bean trypsin hydrolysates (IC_{50} of 406 µg protein/mL) and pinto and dark red kidney GIS hydrolysates (IC_{50} of 198 µg protein/mL and 199 µg protein/mL, respectively).

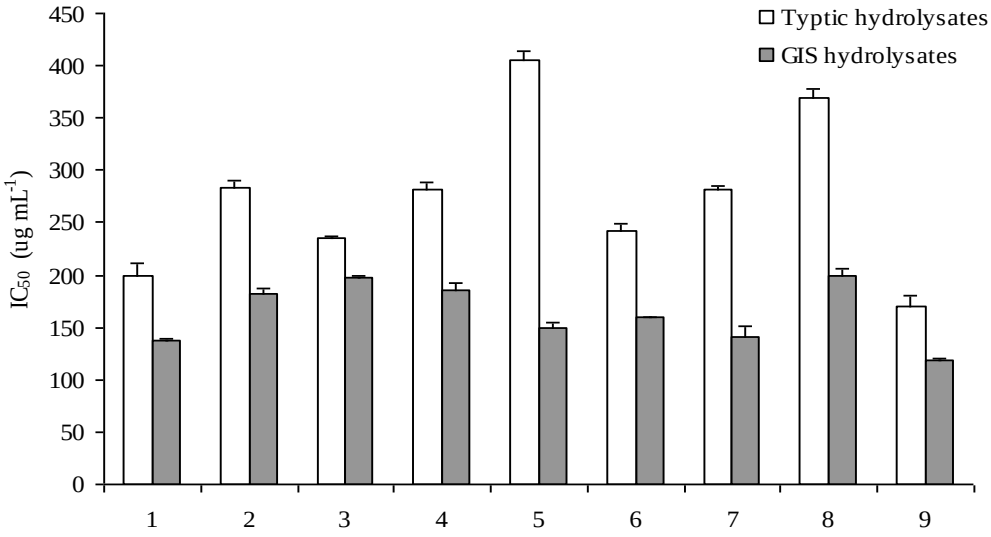


Fig. 4-2. IC_{50} values for ACE inhibition properties of bean protein hydrolysates.
1- navy; 2- pink; 3- pinto; 4- cranberry; 5- black; 6- great northern; 7- light red kidney; 8- dark red kidney; 9- small red.

4.3.4 Time-course electrophoresis of selected dry bean varieties

To further understand changes occurring during the hydrolysis treatment, samples from the navy, black, and small red bean digests were collected at different time intervals during the digestion and subjected to electrophoresis (Fig. 4-3a, b, c, d, e and f). The three varieties were chosen based on the ACE inhibitory results, which showed navy and small red bean hydrolysates to have high ACE inhibitory activity, and marked improvement in ACE inhibitory activity of black bean hydrolysates after GIS treatment compared to the trypsin digestion.

Minor differences were observed in the electrophoretic profiles with black bean protein isolates having intense bands in the high MM range and missing bands in the 60 to 97 kDa MM range (lane 0 min, Fig. 4-3b). After addition of trypsin, proteins having MM higher than 30 kDa from all the investigated samples degraded within 5 min (Fig. 4-3a, b and c) leaving only one persistent faint band with MM of 45 kDa. Two new bands, with MM of around 24 kDa (major) and 28 kDa (minor) appeared which did not degrade any further even after 120 min of hydrolysis (Fig. 4-3a, b and c).

The electrophoretic profiles of the samples subjected to GIS treatment

were very similar (Fig. 4-3d, e and f). As expected, pre-treatment with α-amylase did not change the SDS-PAGE profiles (lane 1–2, Fig. 4-3d, e and f). Addition of pepsin had little impact on the 47 kDa protein (lane 3–8, Fig. 4-3d, e and f). However, it led to the degradation of the proteins with MMs of 20 kDa, 33 kDa, and higher (>50 kDa). New bands with estimated MM of 22 kDa were generated for navy and small red bean hydrolysates (lane 3–8, Fig. 4-3d and f). Subsequent treatments with trypsin and α-chymotrypsin increased the intensity of the 24 kDa and 28 kDa proteins for all varieties (lane 9–14, Fig. 4-3d, e and f), whereas the newly formed proteins with MM around 22 kDa generated after the addition of pepsin were gradually degraded to smaller peptides with the addition of typsin and α-chymotrypsin (lane 9–14, Fig. 4-3d and f).

4.4 Discussion of Results

Dry bean proteins are known to be resistant to proteolysis (Sathe, 2002). Various workers have indicated that the predominant protein in dry beans, i.e. phaseolin, which has a MM of around 47 kDa, has a compact structure and high β-sheet conformation which makes it resistant to peptic digestion and partially resistant to trypsin and α-chymotrypsin digestion (Liener & Thompson, 1980; Momma, 2006; Rui et al., 2011). This is consistent with our findings. The degree of hydrolysis (DH) value, which represents the extent of hydrolysis, was very low for all investigated varieties using both methods of digestion. Previous researches (Barbana & Boye, 2010; Barbana & Boye, 2011) have reported almost three times higher DH values i.e. 27.08% – 40.78% for other pulses such as chickpea, pea and lentil subjected to similar GIS digestion. The results, therefore, suggest that the bean samples analyzed in this study are more resistant to digestion than some of these other pulses. However, the limited degree of hydrolysis did not prevent the enzymatic release of ACE inhibitory peptides from the intact proteins. All investigated dry beans from both digestion treatments exerted comparable *in vitro* ACE inhibitory activities to some pulses hydrolysates, such as chickpea protein GIS digests (IC_{50}:140 – 229 μg/mL) (Barbana & Boye, 2010), and pea

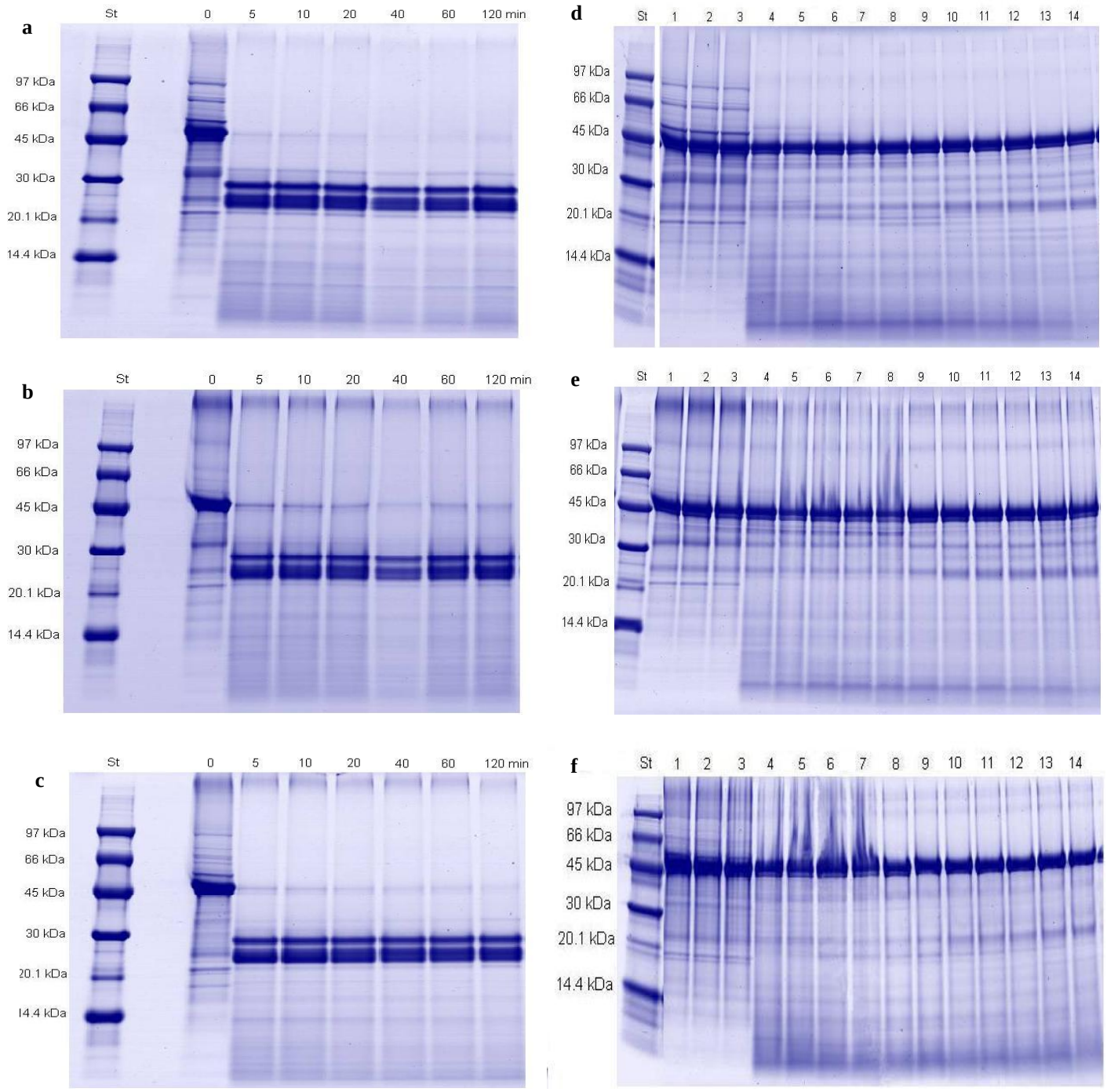


Fig. 4-3. SDS-PAGE of bean protein hydrolysates after different time intervals of trypsin hydrolysis (a-navy, b-black, and c-small red); and *in vitro* gastrointestinal simulation (GIS) digestion hydrolysates (e-navy, f-black, and g-small red) under reducing conditions (5% β-ME).

lane 1: 0 min; lane 2: 3 min after α-amylase digestion; lanes 3 to 8: 0 min, 5 min, 10 min, 30 min, 60 min, 120 min of peptic digestion, respectively; lanes 9 to 14: 0 min, 5 min, 10 min, 30 min, 60 min, 150 min of trypsin/α-chymotrypsin digestion, respectively.

protein GIS digests (IC_{50}: 70 – 159 µg/mL) (Barbana & Boye, 2010; Vermeirssen et al., 2005). However, the ACE inhibitory activities were less than those reported for lentil protein trypsin digests (IC_{50}: 111 µg/mL) and GIS digests (IC_{50}: 53 – 90 µg/mL) (Boye et al., 2010; Barbana & Boye, 2011).

Different enzymatic treatments had varying impacts on the hydrolysate profiles and the ACE inhibitory properties of the dry beans. The SDS-PAGE patterns indicated trypsinolysis of phaseolin occurred very quickly and produced peptides with MM half of the original sizes. This phenomenon, which is in agreement with other previous studies (Nielsen et al., 1988; Romero & Ryan, 1978), may be explained by the presence of a hydrophilic region located near the center of phaseolin which is easily accessible for proteases to attack (Nielsen et al., 1988). Interestingly, phaseolin was hardly degraded during GIS digestion probably due to the lower enzyme to substrate (E/S) ratios used in the GIS digestion, i.e. 1:250 compared to 1:25 used for trypsinolysis. Previous studies demonstrated minor degradation of phaseolin during trypsinolysis using an E/S ratio of 1:100; at a higher E/S ratio of 1:10 phaseolin was halved in 3 min (Nielsen et al., 1988; Deshpande & Damodaran 1989b). Compared to trypsin digestion, the significantly higher DH values ($P<0.05$) of the GIS digestion suggest that more small peptides were released during the GIS digestion. This was reflected in the improvement of the ACE inhibitory activities for all investigated dry bean samples after GIS treatment.

Of all the investigated dry bean varieties, distinctive peptide profiles were observed for the cranberry and light red kidney bean hydrolysates. The SDS-PAGE profiles suggested that these two bean varieties contained ‘T’ type phaseolin, as categorized by Brown et al., (1981), who demonstrated three phaseolin types, namely, ‘S’, ‘T’, and ‘C’ after the cultivars Sanilac, Tendergreen, and Contender, respectively. The other seven investigated bean varieties showed ‘S’ type phaseolin. It seems, therefore, that dry bean proteins with ‘T’ type phaseolin had higher proteolytic susceptibility to both digestion treatments, based on the high

DH values obtained for cranberry and light red kidney bean hydrolysates as well as the intensive bands observed on the SDS-PAGE profiles at the lower MM range. Digestibility differences between various types of phaseolin were reported recently (Montoya et al., 2005). In that study, 'T' type phaseolin showed higher DH values than that of 'S' type phaseolin. Interestingly, the cranberry and light red kidney bean hydrolysates obtained from both digestion treatments did not yielded significantly higher ACE inhibitory activities compared to the other seven varieties, even though they seemed to have contained more small peptides.

Black bean was particularly different among the samples. DH value of black bean hydrolysates from GIS digestion was 5.4 times higher than that of the trypsin digestion, which was the highest among all the samples. Moreover, the differences between IC_{50} values from the two digestions were also the highest for black bean, i.e. 2.7 times. We speculate that this might be due to the more intensive degradation of large protein aggregate in black bean after GIS digestion and the consequent liberation of larger quantities of small peptides as shown on the SDS-PAGE profiles.

A comparison between the digestibility and ACE inhibitory activity values of the dry bean varieties studied showed no obvious relationship. For example, small red bean, which had the highest ACE inhibitory activities of all investigated samples, had average DH values and similar SDS-PAGE profiles compared to the other varieties for both digestion treatments. Peptides need to meet several criteria in order to yield ACE inhibitory properties, such as shape, molecular mass, hydrophobicity, charge and electronic properties (Hernández-Ledesma et al., 2011). They are expected to be short, i.e. 2 – 12 amino acids (Hernández-Ledesma et al., 2011) and more likely to have hydrophobic amino acids as C-terminal residue, such as tryptophan, tyrosine, phenylalanine and proline (Cheung et al., 1980). Thus, the ACE inhibitory activities of the dry bean hydrolysates analyzed in this study may have been influenced not only by the extent of hydrolysis but also other factors, such as parent protein composition,

structure, sequence and enzymatic digestion mechanisms (Barbana & Boye, 2010).

4.5 Conclusions

In conclusion, the current study investigated for the first time the *in vitro* ACE inhibitory properties of protein hydrolysates obtained from nine largely grown Canadian *Phaseolus vulgaris* bean varieties. We demonstrated that: a) all investigated bean varieties had ACE inhibitory activities when subjected to both trypsin and *in vitro* GIS digestion treatments; b) all investigated varieties showed significantly higher ACE inhibitory activities after GIS digestion than after trypsinolysis; and c) there were no obvious relationship between dry bean digestibility and ACE inhibitory activity. Overall, small red bean demonstrated the highest ACE inhibitory activity and should be the focus for any future *in vivo* studies.

Connecting Statement to Chapter 5

In chapter 4, ACE inhibitory properties were investigated for all nine dry bean varieties. Dry bean protein digests from both tryptic and *in vitro* gastrointestinal digestions showed high ACE inhibitory activity which suggested that dry bean proteins may be good sources for producing antihypertensive peptides. It was, however, observed that the major dry bean protein, phaseolin, was resistant to both digestion treatments; thus, the release of bioactive peptides from this protein, if present, may be prevented. It was hypothesized that the employment of preheat treatment and the use of a combination of different commercial enzyme(s) will help in enhancing the hydrolysis of the dry bean proteins. Therefore, in chapter 5, dry bean proteins were subjected to preheat treatment followed by hydrolysis using sequential digestions of Alcalase/Flavourzyme or Alcalase/papain, and the results were compared with samples without preheat treatment. Three varieties, namely, navy, black, and small red bean were selected for this study either because they showed one of the highest ACE inhibitory activities in the previous chapter (navy and small red bean), or they presented the most significant difference of ACE inhibitory activity between tryptic digestion and *in vitro* gastrointestinal digestion (black bean). The results of this study have been presented at the following conference and being prepared as a paper to be submitted to the Food Research International Journal as follows:

Rui, X., Boye, J. I., Simpson, B. K., & Prasher, S. O. (2012) Angiotensin I-converting enzyme inhibitory properties of *Phaseolus vulgaris* bean hydrolysates: Effects of heat treatments and different enzymatic digestions. *Food Research International* (accepted).

Rui, X., Boye, J. I., Simpson, B. K., & Prasher, S. O. (2012) Angiotensin I-converting enzyme inhibitory properties of *Phaseolus vulgaris* bean hydrolysates: Effects of

thermal and different enzymatic digestion treatments. IFT 2012 Annual Meeting, June 25th–28th, Las Vegas, NV, USA (poster presentation).

Chapter 5: Angiotensin I-converting enzyme inhibitory properties of *Phaseolus vulgaris* bean hydrolysates: Effects of heat treatments and different enzymatic digestions

Abstract

ACE inhibitory activity was investigated for protein hydrolysates derived from protein isolates of three *Phaseolus vulgaris* varieties, namely, navy bean, black bean and small red bean. Hydrolysis was performed using sequential digestion of Alcalase/Flavourzyme (AF) or Alcalase/papain (AP). Samples of the protein isolates were heated prior to hydrolysis and the results (i.e., degree of hydrolysis (DH), electrophoresis profiles, and ACE inhibitory activities) were compared to unheated samples. For all varieties, significantly higher DH values ($P<0.05$) were obtained for the AF hydrolysates compared to the AP hydrolysates. Heat treatments improved DH values for navy bean and small red bean, however, lower values were observed for the heated black bean samples. For all unheated samples, phaseolins were degraded slowly to fragments with estimated molecular mass around 20–24 kDa which were resistant to further AF or AP digestions. Extensive degradations were observed for heat-treated samples and phaseolins were hydrolyzed into smaller peptides at the initial stage of digestions. AP hydrolysates for all investigated varieties presented higher ACE inhibitory activity than the AF hydrolysates. The highest ACE inhibitory activities were obtained after 100 min of AP digestion for heated samples from navy bean, and 95 min of AP digestion for heated samples from both black and small red bean, which yielded IC_{50} of 68±5 μg protein/mL, 83±13 μg protein/mL and 78±7 μg protein/mL, respectively. Stabilities of these hydrolysates were tested by simulated *in vitro* gastrointestinal digestion followed by ACE inhibition studies, and lower IC_{50} values were determined for all three hydrolysates. The results obtained should be useful in optimizing conditions for the production of ACE

inhibitory peptides from dry beans.

5.1 Introduction

Dry bean (*Phaseolus vulgaris* L.) is a staple food in the human diet. Canada produces a large volume of dry beans and is also one of the major leaders in global bean exportation. Canada was ranked as one of the world's top five dry-bean exporters during the 10 years from 1999 to 2009 (Food and Agriculture Organization of the United Nations, 2011). Dry beans are rich in fiber, vitamins, minerals, and low in fat (Berrios, 2006). They are also high in protein. Most dry bean varieties contain 20-30% protein on a dry weight basis, which is twice as much as what is found in cereal grains (7-14%). When consumed with cereals, they provide lysine which is often lacking in cereal grains (Gupta, 1982).

Phaseolins, the major storage proteins in dry beans, which present up to 50% of the total seed proteins, have low digestibility when hydrolyzed with pepsin, trypsin, chymotrypsin and several other commercial enzymes (Deshpande & Nielsen, 1987b; Lawrence et al., 1994). Additionally, the presence of several protease inhibitors and lectins limit the nutritional value of dry bean protein. As a result, consumption of dry bean as whole seed requires several pretreatments (e.g., soaking, blanching and boiling) (Khattab et al., 2009) to increase its nutritional value.

In recent years, bioactive peptides have been identified in some proteins which enhance their beneficial properties beyond that of basic nutrition. Many studies have demonstrated that small peptides derived from parent proteins, either from animal or plant sources can exert bioactive properties such as antimicrobial activity, blood pressure-lowering effects, antioxidant properties, and antithrombotic activities (Hartmann & Meisel, 2007). Peptides delivering antihypertensive properties have particularly received increased attention, because hypertension affects about 26.4% of the world's adult population, and is

a highly independent risk factor for cardiovascular disease, which is the leading cause of death at the present time (Kearney et al., 2005).

The antihypertensive activities of bioactive peptides are predominantly related to the inhibitory effects of Angiotensin I-converting enzyme (ACE). ACE is a critical enzyme in the human blood pressure regulatory pathway, which is responsible for converting the decapeptide angiotensin I to the octapeptide angiotensin II, a potent vasoconstrictor; it also degrades bradykinin, a vasodilator, into inactive peptides (Yang & Erdös, 1967; Yang et al., 1970). This dual role enables the enzyme to elevate blood pressure.

Adverse side effects of synthesized ACE inhibitory drugs (e.g., cough, exanthema, taste alterations, skin rashes, gastric problems and edema of the lips) have focused attention on the identification and extraction of ACE inhibitors from natural food sources in order to limit side effects and curtail reliance on synthetic compounds (Torruco-Uco et al., 2009; Vyssoulis et al., 2001). To date, ACE inhibitory peptides have been identified in pulses, such as chickpea (Pedroche et al., 2002; Yust et al, 2003; Barbana & Boye, 2010), pea (Barbana & Boye, 2010; Vermeirssen et al., 2004; Vermeirssen et al., 2005), and lentil (Boye et al., 2010; Barbana & Boye, 2011). However, very few studies have investigated dry bean proteins for the presence of ACE inhibitory peptides.

In this study, we examine the ACE inhibitory properties of proteins extracted from selected dry bean varieties that are largely grown in Canada, namely, navy bean, black bean and small red bean, and propose the use of heat treatment and a two sequential digestion processes, namely Alcalase/Flavourzyme (AF) and Alcalase/papain (AP) to enhance the liberation of ACE inhibitory bioactive peptides. The specific objectives of this study, therefore, were as follows: 1) Compare the effectiveness of AF and AP digestion in generating ACE inhibitory peptides from three varieties of dry bean protein isolates; 2) Determine the impact of pre-heat treatment on the digestibility and ACE inhibitory properties of the bean protein isolates; and 3) Determine the

effect of *in vitro* gastrointestinal digestion simulation on the ACE inhibitory properties of the hydrolysates.

5.2 Materials and methods

5.2.1 Materials

Three varieties of dry beans, namely navy bean, black bean, and small red bean were kindly provided by Pulse Canada (Winnipeg, MB, Canada) and were stored at 4 °C until used.

Alcalase 2.4 Anson units (AU) g^{-1} and Flavourzyme 1000 MG were from Novozyme (Bagsvaerd, Denmark). Papain (P 4762), pepsin (P 6887), trypsin (T 0303), α-chymotrypsin (C 4129), Angiotensin I-converting enzyme (ACE) regent (A 6778), Hippuryl-His-Leu (HHL) (H 1635) were purchased from Sigma-Aldrich Co. (Oakville, ON, Canada). Low-molecular mass calibration kits were from Amersham Pharmacia Biotech (GE Uppsala, Sweden). Kaleidoscope polypeptide standards were from Bio-Rad Laboratories (Mississauga, ON, Canada). All other reagents used were of analytical grade.

5.2.2 Protein extraction

Protein isolates from navy bean, black bean, and small red bean were prepared as described in Rui et al., (2011).

5.2.3 Protein analysis

Protein/peptide contents in both protein isolates and hydrolysates were determined as indicated in 4.2.2.

5.2.4 Protein hydrolysis

Protein hydrolysis using sequential digestions with Alcalase/Flavourzyme (AF), and Alcalase/papain (AP) were conducted on a pH-stat apparatus (TIM 865, Radiometer Analytical SAS, Villeurbanne, France) under controlled conditions for pH, temperature, and stirring speed. Suspensions of protein isolates from navy

bean, black bean and small red bean were prepared using phosphate buffer (0.01 M, pH 7.0) at 2.5% (w/v). Samples undergoing pre-heat treatments were prepared by heating the protein isolate suspensions in boiling water for 15 min prior to hydrolysis. AF sequential digestions were performed as previously described in Pedroche et al. (2002) with slight changes. Briefly, Alcalase was added at an enzyme/substrate ratio (E/S) of 1/8 (w/w, based on protein content of the isolates) and incubated at 50 °C for 1.5 h. Subsequently, the pH was adjusted to 8 with 1 M NaOH. Flavourzyme was added using E/S 1/10 and hydrolysis was carried out at 50 °C for 1.5 h. AP hydrolysis was started by addition of Alcalase using the same condition as previously described. The mixture was then adjusted to pH 6.5 using 1 M HCl and papain was added at E/S 1/25, and digested for 1.5 h at 60 °C. Reactions were terminated by heating samples at boiling temperature for 10 min. The hydrolysates were then centrifuged at 12,000 *g*, 4 °C for 20 min to remove any precipitate. Supernatants were collected, stored at -80 °C and freeze dried. At different time intervals during both digestions, 200 µL hydrolysates were withdrawn, boiled for 10 min, filtered through 0.45 µm filters (Phenomenex, Torrance, CA, USA) and frozen immediately at -80 °C for ACE inhibitory study. Similarly, 5 µL aliquots were taken, mixed with 45 µL tricine buffer, boiled for 10 min and subjected to electrophoretic studies.

5.2.5 Degree of hydrolysis

Degree of hydrolysis (DH) was determined with the pH-stat method described in Adler-Nissen (1986) using the following formula:

$$DH\% = 100\ BNb(1/\alpha)(1/MP)(1/htot)$$

where B refers to base consumption (mL) during digestion, Nb is the normality of the base, α is the average degree of dissociation of the α-NH2 groups, MP is the protein mass in the starting material (g), and htot is the total number of peptide bonds in the protein substrates (meqv/g protein). Average

weight of 113 for amino acid residues was used for calculation of the total number of peptide bonds in the protein (Ahn et al., 1991).

5.2.6 Electrophoresis

Bean protein hydrolysates from unheated and heated protein isolates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was carried out under reducing conditions (presence of 5% β-mercaptoethanol [β-ME]) by using 10-20% gradient polyacrylamide Tris-HCl gels and 16.5% Tris-tricine gels, respectively. SDS-PAGE was conducted on a Bio-Rad Criterion Cell (Bio-Rad Laboratories, Inc., Mississauga, ON, Canada), with constant voltage 200 V and 100 V for Tris-HCl and Tris-tricine gels, respectively. Low-molecular mass (14.4–97 kDa) calibration kits from Amersham Pharmacia Biotech (GE Healthcare Biosciences, Uppsala, Sweden) and Kaleidoscope polypeptide standard (3.578–31.668 kDa) from Bio-Rad Laboratories (Mississauga, ON, Canada) were used as molecular mass standards for Tris-HCl SDS-PAGE and Tris-tricine SDS-PAGE, respectively. Gels were scanned with Image Scanner III (GE Healthcare Biosciences, Uppsala, Sweden) and then analyzed using Image Quant TL version 2005 software (GE Healthcare Biosciences, Uppsala, Sweden).

5.2.7 Angiotensin I-converting enzyme inhibitory properties

ACE inhibitory activity assay was performed as described in 4.2.6.

5.2.8 *In vitro* gastrointestinal digest simulation test

Simulation of human gastrointestinal digestion (GIS) was performed *in vitro* by sequential digestion using pepsin, trypsin and α-chymotrypsin and a pH-stat apparatus (TIM 865, Radiometer Analytical SAS, Villeurbanne, France) (Vermeirssen et al., 2005). Freeze-dried bean protein hydrolysates were resolubilized in glycine buffer (0.01 M, pH 2.0) at 2.5% (w/v). Pepsin digestion was performed with E/S 1⁄250 (w/w) at 37 °C for 2 h, followed by trypsin and α-chymotrypsin digestion with E/S 1⁄250 (w/w) at 37 °C for 2.5 h during which time

the pH was adjusted to 6.5 with 1 M NaOH. Digestions were stopped by heating samples in boiling water for 10 min. Samples were stored at -80 °C and then freeze-dried for ACE inhibitory study.

5.2.9 Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple comparison tests were used to determine the significant differences between means ($P < 0.05$) using SAS Server Interface (version 2.0.3, SAS Institute, Cary, NC).

5.3 Results and discussion

5.3.1 Enzymatic hydrolysis

The degree of hydrolysis (DH) as a function of hydrolysis time during the digestion with Alcalase/Flavourzyme (AF), and Alcalase/papain (AP) are presented in Fig. 5-1. The first 90 min of digestion was done only using Alcalase. Alcalase digestion generated appreciable DH values for all investigated samples. At the end of the Alcalase treatments, a DH of 17.11% was obtained for navy bean hydrolysate without pre-heat treatment which was significantly higher ($P<0.05$) than that of black bean (15.39%) and small red bean (13.90%). Similar results were observed for the pre-heat treated samples with navy bean hydrolysate showing the highest ($P<0.05$) DH of 33.20%, followed by small red bean (23.53%) and black bean (8.05%). A significant improvement in DH upon heat treatment was, thus, observed for navy bean and small red bean as discussed further below.

Sequential digestion with Flavourzyme following Alcalase treatment improved DH values than that with papain treatment for all investigated samples. This difference is likely due to the different modes of action of the two enzymes. Alcalase is an endoprotease with broad enzymatic specificities which liberates small peptides and thus increases the numbers of terminal amino acid residues in

the hydrolysates. This may facilitate the sequential digestion with Flavourzyme, an exopeptidase (Pedroche et al., 2002), than that for papain, an endoprotease.

For protein isolates without pre-heat treatment, navy bean AF hydrolysates showed DH of 24.01% at the end of the 3 h digestion, which is significantly higher ($P<0.05$) than that of black bean AF hydrolysates (22.09%) and small red bean AF hydrolysates (19.78%), and which is indicative of extensive hydrolysis. This value is, however, very low compared to data reported by Barbana and Boye (2010; 2011) for other pulses species such as chickpea, yellow pea, and lentil subjected to similar AF digestions, i.e. 58.56% to 78.43%. Phaseolins, the major proteins in dry beans, have a compact structure and molecular structural constraints may have made many susceptible sites inaccessible to hydrolysis (Romero & Ryan, 1978; Chang & Satterlee, 1981). Deshpande and Damodaran (1989b) in later studies also showed that native pea vicilin had higher susceptibility towards trypsin than native dry bean phaseolin.

Pre-heat treatment facilitated the hydrolysis for navy bean and small red bean protein isolates by increasing DH values by almost two fold (Fig. 5-1a and c). Thermal treatment, however, decreased DH values for black bean protein isolates during all digestion treatments (Fig. 5-1b). It is well known that heating can alter the tertiary and quaternary structures of phaseolin which can lead to overall improvements in its susceptibility to proteases (Deshpande & Nielsen, 1987b; Deshpande & Damodaran, 1989a). We hypothesize that the decrease in DH of the heated black bean sample might be related to extensive formation of protein aggregates induced by heat as observed in the electrophoretic profiles discussed below.

5.3.2 Electrophoresis

Protein hydrolysis was monitored during both the AF and AP digestion processes using SDS-PAGE. Figs. 5-2 and 5-3 showed the electrophoretic profiles for samples pretreated with and without heat, respectively. The three dry bean protein isolates had some similarities with all having major bands at around

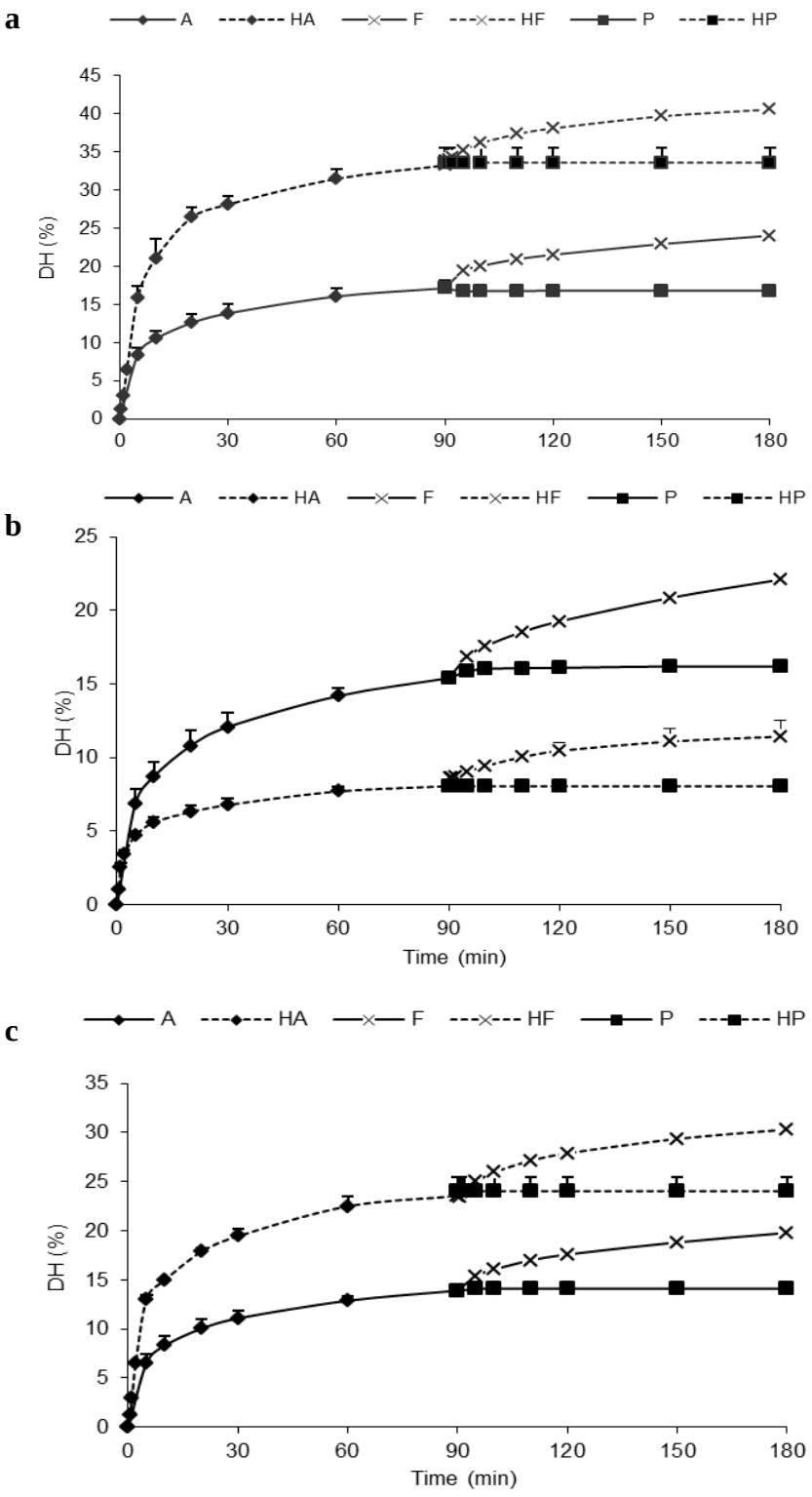


Fig. 5-1. Enzymatic hydrolysis of (a) navy bean, (b) black bean, and (c) small red bean. The experiments were performed in duplicate.

HA: Alacalse digestion using samples with pre-heat treatment; HF: Flavourzyme digestion using samples with pre-heat treatment; HP: Papain digestion using samples with pre-heat treatment; A: Alcalase digestion; F: Flavourzyme digestion; P: papain digestion.

molecular mass (MM) of 45 kDa; there were, however, several differences in their profiles as shown in lane 1 of Figs. 5-2a, b and c. Navy bean protein isolates showed no bands at the top of the lane (lane 1, Fig 5-2a), whereas black and small red bean showed the presence of large proteins or protein aggregates which failed to enter into the gel (lane 1, Fig. 5-2b and c). Additionally, black bean was missing bands in the 60 – 97 kDa MM range (lane 1, Fig. 5-2b), whereas small red bean showed intensive bands at MM of 36, 27, 24, 21, and 18 kDa (lane 2, Fig. 5-2c).

With the exception of phaseolin, other proteins present in the electrophoregrams of the unheated protein isolates were readily degraded to smaller fractions within 5 min of Alcalase digestion (lane 2, Fig. 5-2a, b and c). Complete degradation of phaseolin was only achieved after 30 min treatment with Alcalase for navy bean (lane 5, Fig. 5-2a), and 1 hr for black bean and small red bean (lane 6, Fig. 5-2a and b). The major hydrolysis products for all unheated samples had estimated MM ranging from 20 – 24 kDa, and were considered to be primarily degradation products from phaseolin (Bradbear & Boulter, 1984; Deshpande & Nielsen, 1987b; Liener & Thompson, 1980). The approximately half reduction of the original size of phaseolin may be explained by the fact that the central region of phaseolin is more susceptible to proteases (Deshpande & Nielsen, 1987b). Further hydrolysis with Alcalase and addition of Flavourzyme or papain, induced no further degradation of these fractions (lane 8 – 19, Fig. 5-2a, b and c). Resistance of these degraded fractions to several other proteases, such as trypsin, α-chymotrypsin, and protease E has been reported (Deshpande & Nielsen, 1987b; Deshpande & Damodaran, 1989a).

Various small polypeptides under 14 kDa were also observed in the electrophoregrams for all unheated samples from the initial stages of digestion. As the hydrolysis continued, the decrease in the intensity of these bands and disappearance of some bands suggested further degradation of the peptides into much smaller fractions. Alcalase as well as AF sequential digestion has proven

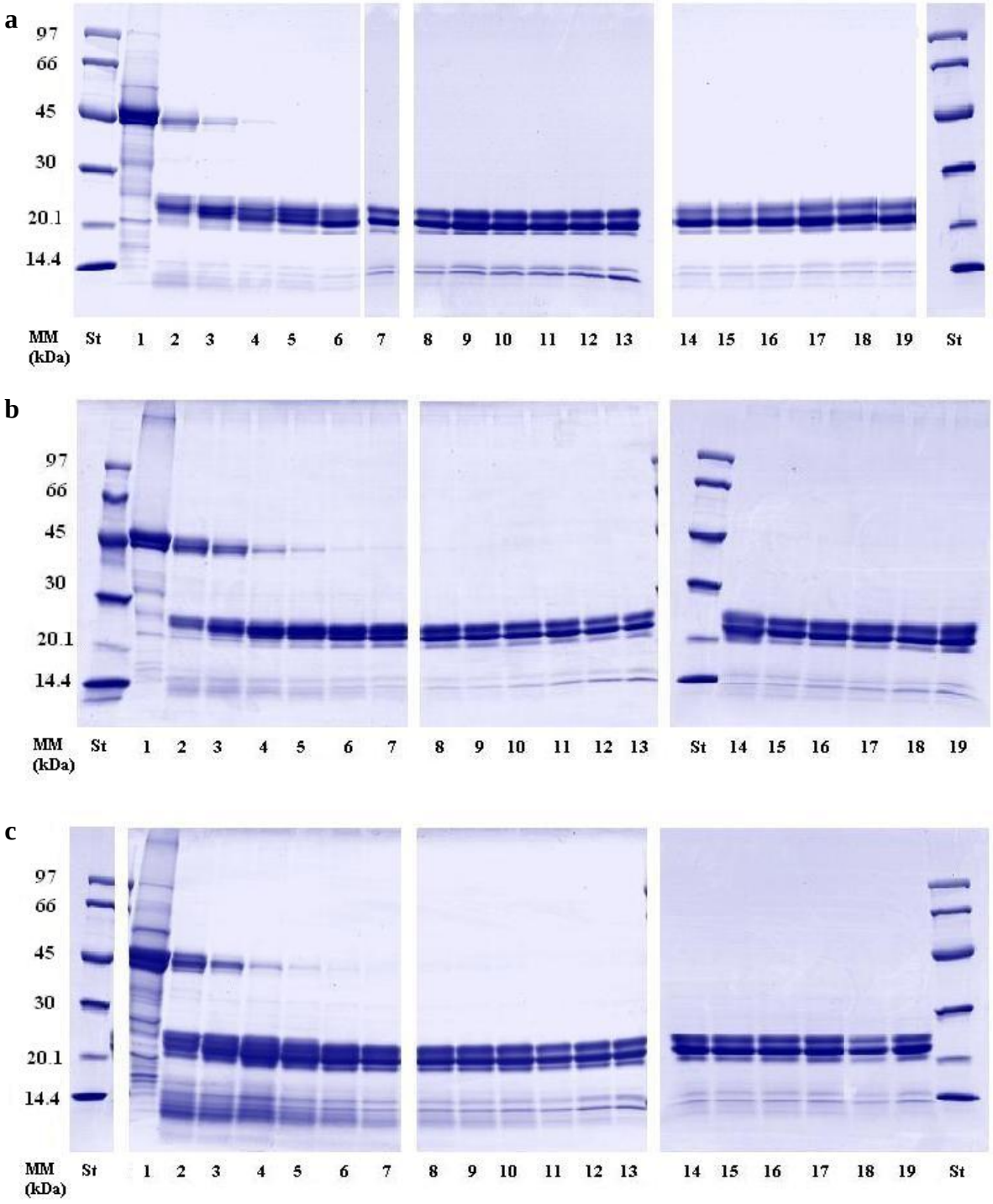


Fig. 5-2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of bean protein hydrolysed for different periods. (a) navy bean, (b) black bean, and (c) small red bean.

Lane 1-7: 0 min, 5 min, 10 min, 20 min, 30 min, 60 min, 90 min of Alcalase digestion. Lane 8-13: 5 min, 10 min, 20 min, 30 min, 60 min, 90 min of the sequential Flavourzyme digestion. lane 14-19: 5 min, 10 min, 20 min, 30 min, 60 min, 90 min of the sequential papain digestion.

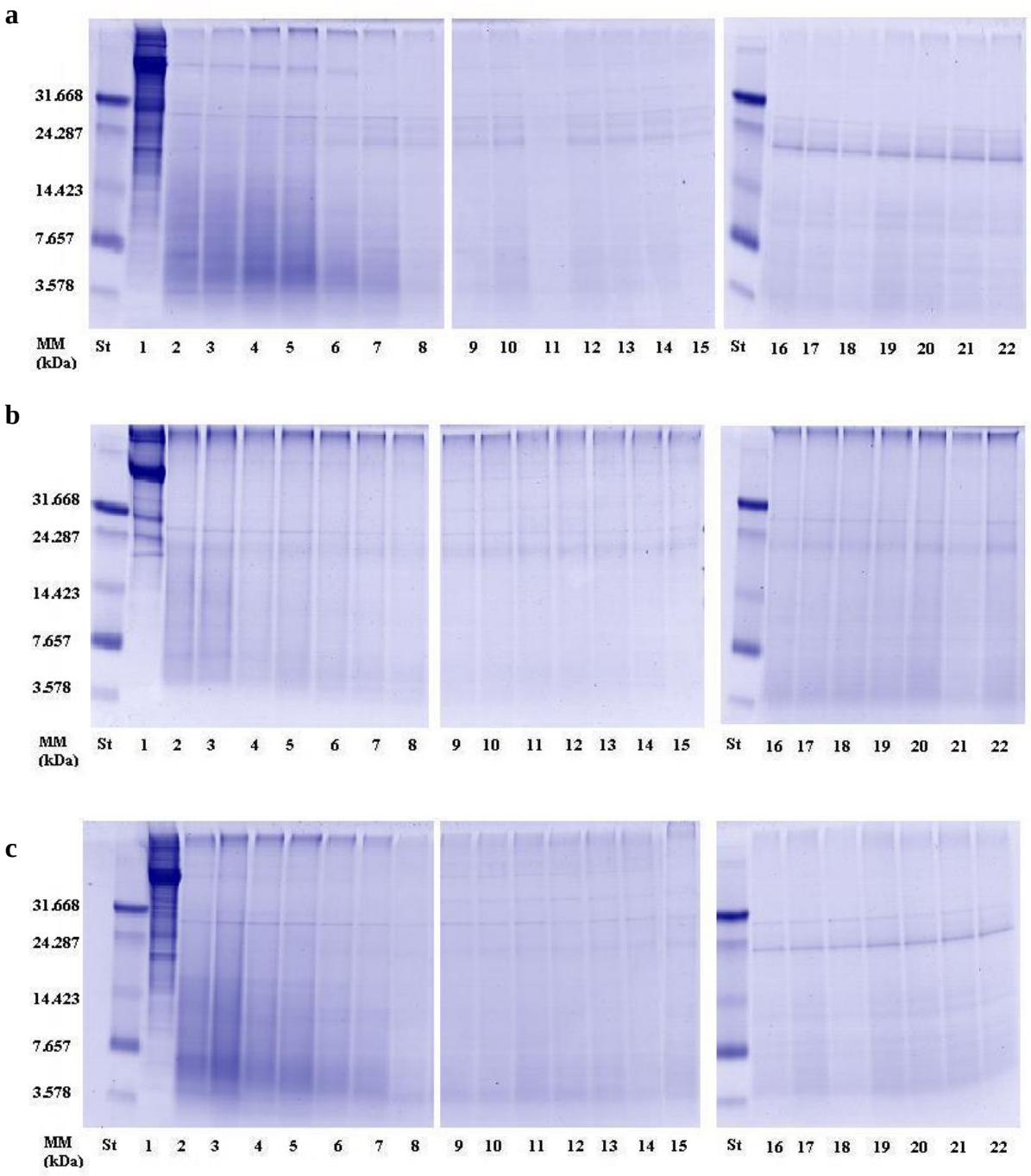


Fig. 5-3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of preheat-treated bean proteins hydrolysed for different periods. (a) navy bean, (b) black bean, and (c) small red bean.

Lane 1-8: 0 min, 0.5 min, 1 min, 2 min, 5 min, 10 min, 30 min, 90 min of Alcalase digestion. Lane 9-15: 0.5 min, 1 min, 2 min, 5 min, 10 min, 30 min, 90 min of the sequential Flavourzyme digestion. Lane 16-22: 0.5 min, 1 min, 2 min, 5 min, 10 min, 30 min, 90 min of the sequential papain digestion.

efficient in other studies in producing small molecular weight peptides with ACE inhibitory activities from many plant sources, such as chickpea (Barbana & Boye, 2010; Pedroche et al., 2002; Yust et al., 2003), lentil (Barbana & Boye, 2011), lima and jamapa beans (Torruco-Uco et al., 2009). Hydrolysates with ACE inhibitory activities from chickpea Alcalase digests were shown to have molecular masses between 6-14 kDa (Yust et al., 2003).

Pre-heat treatments induced the formation of larger protein aggregates for all of the protein isolates (lane 1, Fig. 5-3a, b and c). Although these aggregates seemed to disappear after 90 min of Alcalase treatments for navy bean and small red bean (lane 1-8, Fig. 5-3a and c), aggregates of black bean were resistant to all three enzyme treatments (Fig. 5-3b). This might explain the lower DH obtained for black bean sample with pre-heat treatment. Pre-heat treatment led to complete degradation of phaseolin within 0.5 min of Alcalase treatment for black and small red bean (Fig. 5-3a and c). Trace amount of phaseolins were observed for navy bean until they were completely degraded after 30 min of Alcalase treatment. Deshpande and Damodaran (1989a) demonstrated marked improvement in the enzymatic susceptibility of phaseolin after thermal treatment which was primarily due to disruption of its tertiary and quaternary structure. This supports the current findings. Phaseolin degraded products, according to the previous reports (Bradbear & Boulter, 1984; Deshpande & Nielsen, 1987b; Liener & Thompson, 1980), were faint persistent bands with MMs of 26 – 27 kDa and 20 – 24 kDa. Several small peptides with MMs ranging below 14 kDa were observed within the first 5 min of Alcalase digestions for navy bean and small red bean hydrolysates (lane 1–5, Fig. 5-3a and c). The digest profile observed for black bean hydrolysates was very different with some faint lower MM bands observed only within the first 1 min of digestion (lanes 2 and 3, Fig. 5-3b). Subsequent treatment with Flavourzyme had little impact on the SDS-PAGE profiles for these samples. Papain digestion, however, increased the

intensity of bands with MM at 21-23 kDa, likely due to increased degradation of larger proteins.

5.3.3 ACE inhibitory activities

ACE inhibitory activities of samples withdrawn at different hydrolysis times during both AF and AP digestions are presented in Fig. 5-4. All samples showed no ACE inhibitory activities before digestion, whereas after 5 min of Alcalase treatments high appreciable amounts of ACE inhibitory activity were observed for all samples, ranged from 43.2% (unheated small red bean) to 60.7% (heated navy bean) (Fig. 5-4a, b and c). The inhibitory activity steadily increased during the following 85 min of Alcalase hydrolysis for all samples except unheated samples of navy bean, which decreased from 50.1% (5 min) to 37.3% (90 min). The broad specificity of Alcalase, as well as its preferential activity at hydrophobic residues, may explain this increase in ACE inhibitory activity since hydrophobic residues especially tryptophan, tyrosine, phenylalaine and proline are reported to be favorable carboxyl terminal amino acid residues for ACE inhibitory peptides (Cheung et al., 1980; Markland Jr. & Smith, 1971).

Subsequent treatments with Flavourzyme led to a decrease of ACE inhibitory activity for all samples (Fig. 5-4a, b and c). The decline was observed immediately after addition of Flavourzyme, which implied that some of the bioactive peptides were degraded which decreased or eliminated their ACE inhibitory activity. This observation may at first seem inconsistent with the results of the DH which increased during the Flavourzyme treatment, and especially since peptides with smaller sizes are believed to have higher ACE inhibitory activity. However, bioactivities of peptides are also largely influenced by terminal amino acid residues as previously illustrated by Cheung et al. (1980). Therefore, it is hypothesized that Flavourzyme which is an exopeptidase, removed some critical terminal amino acids residues from the bioactive peptides generated from the hydrolysis of beans using Alcalase, which may have resulted in the overall reduction of ACE inhibitory activities.

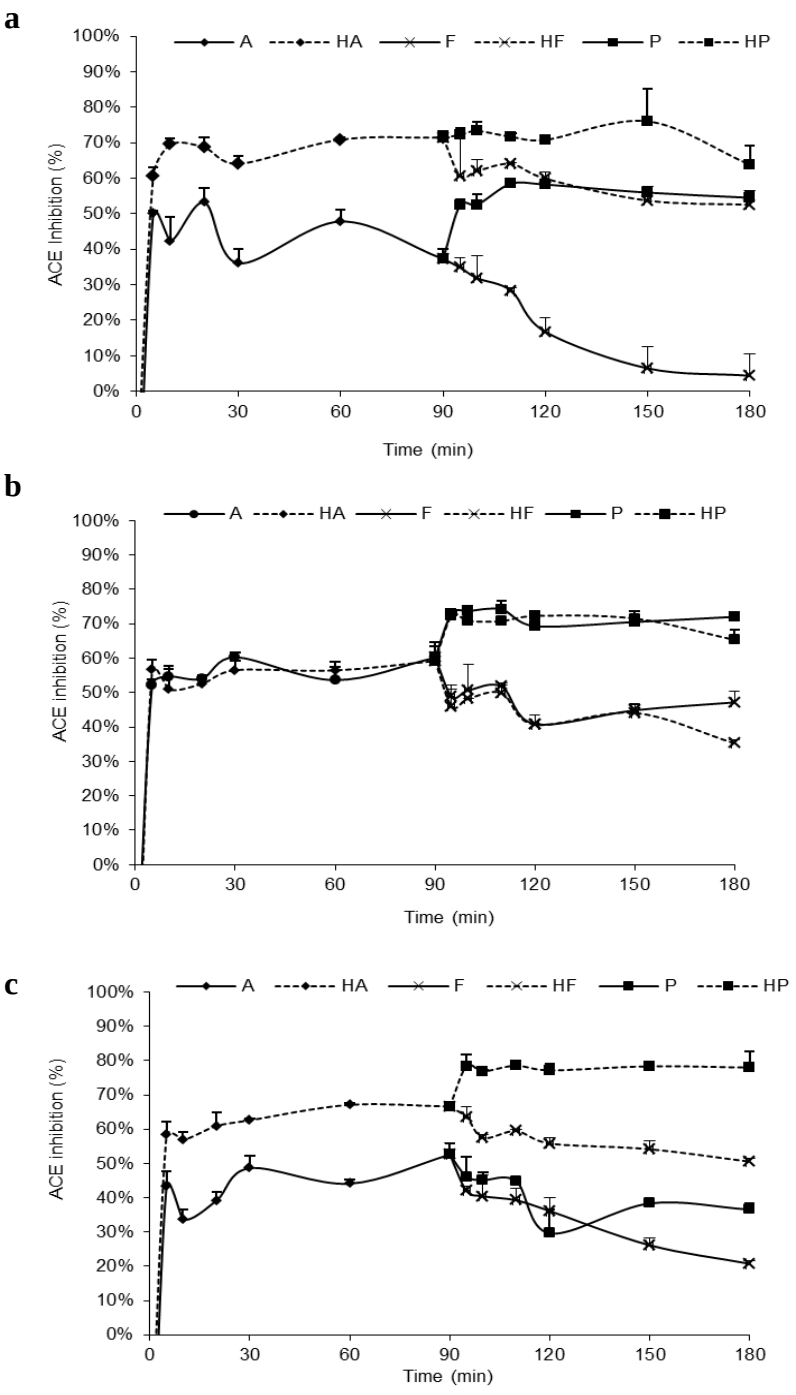


Fig. 5-4. ACE inhibitory activities of bean proteins hydrolysed for different times. (a) navy bean, (b) black bean, and (c) small red bean. Each point represents the average value of four replicates.

HA: Alacalse digestion using samples with pre-heat treatment; HF: Flavourzyme digestion using samples with pre-heat treatment; HP: Papain digestion using samples with pre-heat treatment; A: Alcalase digestion; F: Flavourzyme digestion; P: papain digestion.

Our results are in agreement with a previous study which also used sequential digestion of AF to generate ACE inhibitory peptides from chickpea protein isolates (Pedroche et al., 2002). In this study, ACE inhibitory activity peaked after 30 min of Alcalase treatment, whereas no improvements were observed upon further digestion with Flavourzyme for 90 min.

Interestingly, addition of papain which only showed marginal improvements in DH values, resulted in further liberation of bioactive peptides from the Alcalase hydrolysates of navy bean, black bean and heated small red bean samples (Fig. 5-4a and b). For these samples, ACE inhibitory activities peaked within 20 min of enzymatic treatment and the inhibitory activities were maintained until the end of the treatment. The unheated samples for small red bean presented slightly decreased ACE inhibitory activity for the papain hydrolyzed sample compared to the previously Alcalase hydrolyzed sample (Fig. 5-4c). Overall, compared to Flavourzyme, papain yielded more ACE inhibitory peptides for all samples following Alcalase treatment, which indicated that the specific protease cleavage mechanism of papain may have facilitated the release of ACE inhibitory peptides from bean proteins. Higher ACE inhibitory activities were also found for papain hydrolysates of chickpea, pea, and lentil compared to AF hydrolysates (Barbana & Boye, 2010; Barbana & Boye, 2011).

Pre-heat treatment of bean protein isolates by boiling for 15 min yielded higher ACE inhibitory activities for navy and small red bean compared to the unheated samples (Fig.5-4a and c). These results are consistent with the higher DH values observed and the appearance of abundant small peptides under 14 kDa on the SDS-PAGE gels for these two varieties, indicative of improved susceptibility to enzymatic treatment (likely due to unfolding of the compact structure of the parent proteins). Not surprisingly, no such improvement was found for pre-heat treated black bean hydrolysates (Fig.5-4b) during both AF and AP digestions. This might be related to the presence of large aggregates as shown

by the SDS-PAGE after heating of the protein isolates which probably limited liberation of bioactive peptides.

IC_{50} values were determined for each variety on samples presenting the highest ACE inhibitory activities (i.e., heated samples digested by AP for 100 min, 95 min and 95 min for navy, black and small red bean, respectively). The identified digestion times were chosen because no significant improvements were observed with prolonged hydrolysis time ($P>0.05$). The selected hydrolysates yielded IC_{50} values of 68±5 μg protein/mL, 83±13 μg protein/mL, and 78±7 μg protein/mL for navy, black and small red bean, respectively, with no significant differences among them ($P>0.05$) (Fig. 5-5). Akillioğlu and Karakaya (2009) reported IC_{50} of 770-830, 150-690 μg protein/mL for common and pinto beans, respectively, after thermal treatments at 121 °C for different time periods followed by hydrolysis with gastrointestinal enzymes. The lower IC_{50} values found in the present study suggests that the treatments used in the present study had higher efficiency in releasing bioactive peptides.

5.3.4 *In vitro* gastrointestinal digestion and ACE inhibitory properties of pre-hydrolysed bean peptides

In vivo ACE inhibitory activity expression requires that ACE inhibitory peptides escape from proteolytic digestion during gastrointestinal digestion, and reach the target cell with preservation of their bioactive activities. It is, therefore, important to have information on the resistance of the bioactive peptides to gastrointestinal enzymes. Wu and Ding (2002) proposed a practical way to predict the fate of ACE inhibitory peptides in the gastrointestinal tract by employing in *vitro* digestion with multi gastrointestinal protease combination. Fig.5-5 presents the IC_{50} values of three selected hydrolysates, namely, N (preheat-treated navy bean hydrolyzed for 100 min using Alcalase/papain), B (preheat-treated black bean hydrolyzed for 95 min using Alcalase/papain), and S (preheat-treated small red bean hydrolyzed for 95 min using Alcalase/papain), after being subjected to *in vitro* gastrointestinal simulation (GIS) digestion. Significant reductions of IC_{50}

were observed for N and S ($P<0.01$) compared to that before digestion, with values of 48±4 µg protein/mL, and 39±2 µg protein/mL, respectively, whereas an insignificant decrease ($P>0.05$) was obtained for B (IC_{50}=60±2 µg protein/mL). The difference between the IC_{50} values of S and the other two varieties was found to be significant ($P<0.05$). The results demonstrated that the hydrolysates were either resistant or liberated new ACE inhibitory peptides after gastrointestinal hydrolysis, which suggests that these hydrolysates would probably preserve or demonstrate improved bioactivity in the gastrointestinal tract. Similar results have been reported for soybean Alcalase digests which showed little alteration of IC_{50} values after *in vitro* GIS digestion (Wu & Ding, 2002).

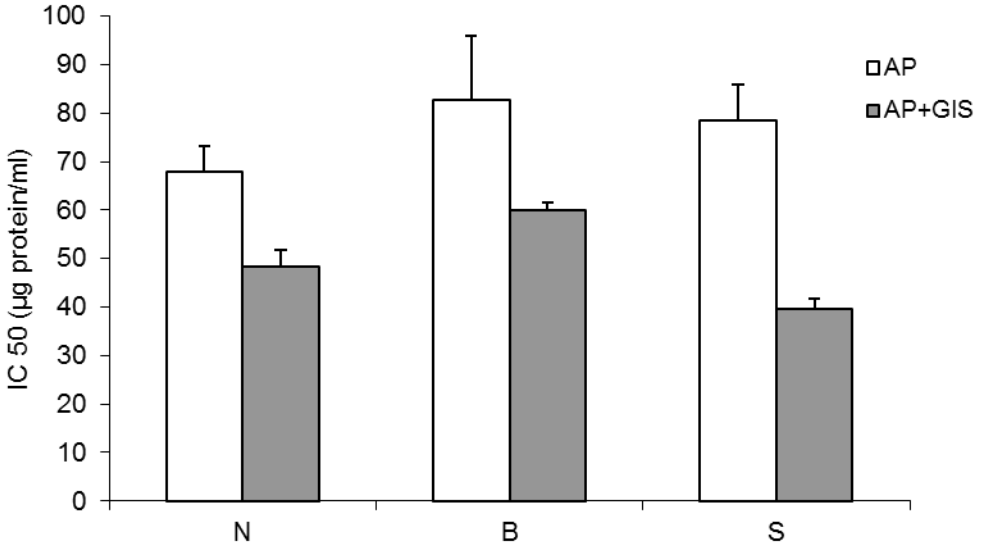


Fig. 5-5. IC_{50} of dry bean protein isolates hydrolysed using optimum conditions.
I.e. N (preheat-treated navy bean hydrolyzed for 100 min using Alcalase/papain digestion), B (preheat-treated black bean hydrolyzed for 95 min using Alcalase/papain digestion), S (preheat-treated small red bean hydrolyzed for 95 min using Alcalase/papain digestion), followed by in vitro gastrointestinal digestion (AP+GIS).

5.4 Conclusions

This study provides novel information of ACE inhibitory properties from navy, black and small red bean hydrolysates produced by Alcalase/Flavourzyme and Alcalase/papain sequential digestions. Results demonstrated that sequential

digestions with Alcalase/papain produced peptides with higher ACE inhibitory activity compared to Alcalase/Flavourzyme. Pre-heat treatment of protein isolates at boiling temperature for 15 min facilitated phaseolin degradation during the enzymatic hydrolysis, and improved the ACE inhibitory activities of hydrolysates from navy bean and small red bean. Higher IC_{50} values observed for hydrolysates subjected to further *in vitro* digestibility simulation, predict a promising fate for these bioactive peptides after oral administration. Overall, this study provides useful information for the potential future utilisation of bean protein hydrolysates as functional ingredient in novel foods targeting hypertension. Further studies are recommended on the *in vivo* behaviour of these hydrolysates as well as their stabilities during processing.

Connecting Statement to Chapter 6

The results from chapter 5 demonstrated that the use of preheat treatment facilitated the hydrolysis of most of the proteins in dry beans. Additionally, the sequential enzyme digestion treatment using Alcalase/papain demonstrated a greater capacity to generate ACE inhibitory peptides compared to that of Alcalase/Flavourzyme. Among the three dry bean varieties investigated, small red bean hydrolysate exerted the highest ACE inhibitory activity when digestion was conducted under optimum conditions. The logical next step was to identify the specific peptide fragments in the hydrolysate responsible for the ACE inhibitory activity of the bean hydrolysate. Furthermore, fractionation and characterization of the active fragments would be useful for studying the inhibition mechanism of ACE, as well as in identifying novel substitutes that could potentially be used as nutraceuticals in antihypertensive treatments, if results are substantiated *in vivo* in any future studies. Therefore, in chapter 6, small red bean protein hydrolysate was prepared following the optimum conditions established in the previous chapter. The peptides were fractioned by sequential application of ultrafiltration, gel filtration and reverse phase chromatography. The most active fraction was collected for sequencing and used to investigate the mode of inhibition. The results of this study are currently being prepared as a paper to be submitted to the Food Chemistry journal as follows:

Rui, X., Boye, J. I., Simpson, B. K., & Prasher, S. O. Purification and Characterization of angiotensin I-converting enzyme inhibitory peptides of small red bean (*Phaseolus vulgaris*) hydrolysates Food Chemistry (submitted).

Chapter 6: Purification and characterization of angiotensin I-converting enzyme inhibitory peptides of small red bean (*Phaseolus vulgaris*) hydrolysates

Abstract

Angiotensin I–converting enzyme (ACE) inhibitory activity was investigated for small red bean (*Phaseolus vulgaris*) protein hydrolysate produced by sequential digestion of Alcalase, papain followed by *in vitro* gastrointestinal simulation. The hydrolysate had ACE inhibitory activity with IC_{50} of 67.2±1.8 µg protein/mL. Size exclusion chromatography demonstrated the hydrolysate was composed of peptides ranging in size from < 0.244 kDa to 7.4 kDa, with the most predominant peptides having a molecular mass of ~ 0.698 kDa. Peptides responsible for potent ACE inhibitory activity were isolated by a three step purification process, including ultrafiltration, gel filtration and preparative reverse phase high performance chromatography (RP-HPLC). Ultrafiltration, performed using a membrane with molecular weight cut-off of 5 kDa, generated a fraction with an IC_{50} value of 50.8±4.4 µg protein/mL. Subsequent gel filtration decreased the IC_{50} value further to 35.1±1.8 µg protein/mL indicating increased ACE inhibitory activity. The most potent fraction was obtained after further fractionation by RP-HPLC which yielded an IC_{50} of 19.3±1.4 µg protein/mL. Enzymatic kinetic studies using this fraction demonstrated competitive inhibition with K_i of 11.6±1.7 µg protein/mL. Mass spectrometric characterization showed with a high probability that this fraction was composed of peptides containing the octapeptide PVNNPQIH. The results presented in the current study expand the knowledge base of ACE inhibitory properties of small red bean protein hydrolysate and should be useful in future purification of ACE inhibitory peptides from dry beans.

6.1 Introduction

Hypertension affects about 26.4% of the world's adult population, and it is also considered as the leading risk factor for mortality (Kearney et al., 2005). Angiotensin I–converting enzyme (ACE) (EC 3.4.15.1) is a key enzyme that regulates blood pressure via the renin-angiotensin system (RAS) (Chen et al., 2009; Erdmann et al., 2008). ACE catalyzes the degradation of the decapeptide angiotensin I to the potent vasoconstrictor angiotensin II by cleavage of a dipeptide at the carboxyl-terminal site (Skeggs et al., 1956). It is also able to degrade bradykinin, a vasodilator, into inactive peptides (Yang & Erdos, 1967; Yang et al., 1970). This dual role enables overall elevation of blood pressure.

Synthetic ACE inhibitors have been used to decrease blood pressure with great success. Captopril was the first ACE inhibitor successfully used in clinical practice (Ondetti et al., 1977). Administration of captopril and other known synthetics, however, has significant adverse effects such as cough, exanthema, taste alterations, skin rashes, gastric troubles and edema of lips (Torruco-Uco et al., 2009; Vyssoulis et al., 2001). Due to these adverse side effects, there is intense interest to identify and extract ACE inhibitors from natural food sources which could serve as substitutes of synthetic compounds.

Dry bean (*Phaseolus vulgaris*), also well known as common bean, is perhaps the most important legume species besides soybean in terms of its economic value (Berrios, 2006). Dry beans form the staple food in many developing and underdeveloped countries such as India, Sri Lanka, Bangladesh; however, in North America (Canada and the US), dry beans remain largely underexploited compared to other legumes, such as soybean and peanut, especially in terms of their bioactive and functional properties (Sathe, 2002). More studies are, thus, needed to identify the beneficial effects of dry bean and its constituents, both in terms of its nutritional and potential health properties, as well as determine different ways in which their use can be expanded.

In previous studies we determined that small red bean protein hydrolysates generated by subsequent digestion of Alcalase, papain and *in vitro* gastrointestinal simulation (GIS) had high ACE inhibitory activity. To expand this knowledge, the current study was undertaken to fractionate the small red bean protein hydrolysate and identify the peptide fractions responsible for the potent ACE inhibitory activity. A three step purification process including ultrafiltration, gel filtration, and reverse phase high performance liquid chromatography (RP-HPLC) was conducted. The fraction having the highest ACE inhibitory activity was further characterized by mass spectrometry and the kinetics of enzyme inhibition was determined.

6.2 Materials and methods

6.2.1 Materials

Small red bean used for this study was kindly provided by Pulse Canada (Winnipeg, MB, Canada) and was stored at 4 °C until used. Alcalase 2.4 Anson units (AU) g^{-1} was from Novozyme (Bagsvaerd, Denmark). Papain (P 4762), pepsin (P 6887), trypsin (T 0303), α-chymotrypsin (C 4129), ACE regent (A 6778), Hippuryl-His-Leu (HHL) (H 1635) were purchased from Sigma-Aldrich Co. (Oakville, ON, Canada). Mixed gel filtration standard was from Bio-Rad Laboratories (Mississauga, ON, Canada). Uridine was from Phenomenex (Torrance, CA, USA). All other reagents used were of analytical grade.

6.2.2 Protein extraction

Protein isolates from small red bean were prepared as described in Rui et al., (2011).

6.2.3 Protein analysis

Protein/peptide contents in protein hydrolysates were determined by measuring the absorbance at 220 nm (Kamizake et al., 2003) using

caseinoglycopeptide as standards. A Cary 300 Bio UV-Visible spectrophotometer was used (Varian Canada, Mississauga, ON, Canada). The standard curve of caseinoglycopeptide concentrations (0.0035 mg/mL to 0.1 mg/mL) showed a linear relationship with r^2= 0.9999.

6.2.4 Protein hydrolysis

Protein hydrolysis using sequential digestions with Alcalase/papain (AP) were conducted on a pH-stat apparatus (TIM 865, Radiometer Analytical SAS, Villeurbanne, France) under controlled conditions for pH, temperature, and stirring speed. Suspensions of small red bean protein isolates were prepared using phosphate buffer (0.01 M, pH 7.0) at 2.5% (w/v). Samples were heated in boiling water for 15 min prior to hydrolysis. The digestion was initiated by addition of Alcalase at enzyme/substrate ratio (E/S) 1/8 (w/w) and was incubated at 50 °C for 1.5 h. The mixture was then adjusted to pH 6.5 by 1 M HCl before papain was added at E/S 1/25 (w/w), and digested for 1.5 h at 60 °C. Reactions were terminated by heating samples at boiling temperature for 10 min. The hydrolysates were then centrifuged at 12,000 *g*, 4 °C for 20 min to remove precipitate. Supernatants were collected to conduct the following *in vitro* gastrointestinal simulation digestion (GIS).

In vitro GIS was performed by sequential digestion using pepsin, trypsin and α-chymotrypsin (Vermeirssen et al., 2005). Pepsin digestion was performed with E/S 1/250 (w/w) at 37 °C for 2 h, followed by trypsin and α-chymotrypsin digestion with E/S 1/250 (w/w) at 37 °C for 2.5 h during which time the pH was adjusted to 6.5 with 1 M NaOH. Digestions were stopped by heating samples in boiling water for 10 min. Samples were stored at -80 °C and freeze dried for ACE inhibitory study.

6.2.5 Characterisation of bean protein hydrolysates by HPLC size exclusion chromatography

HPLC size exclusion chromatography was conducted as described previously (Barbana & Boye, 2011). Small red bean protein hydrolysate was dissolved in 0.1 M phosphate buffer (pH 6.8) at a concentration of 1.6 mg/mL. A Biosep-SEC S2000 column (7.8×300 mm) from Phenomenex (Torrance, CA, USA) connected to a Waters 600 Separation Module System (Water Inc, Mississauga, ON, Canada) was used. 200 µL of the peptide solution was loaded on the column, and eluted with 0.1 M phosphate buffer (pH 6.8) at a flow rate of 0.4 mL/min. The elution was monitored at 280 nm. Mixed gel filtration standards comprising thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B_{12} (1.35 kDa) from Bio-Rad Laboratories (Mississauga, ON, Canada), and uridine (0.244 kDa) from Phenomenex (Torrance, CA, USA) were used as standards to estimate the molecular masses of the samples. The standard curve was plotted of partition coefficient K_{av} against the logarithmic values of molecular masses and linear relationship were found with r^2= 0.9831.

6.2.6 Angiotensin I-converting enzyme inhibitory properties

ACE inhibitory activity assay was performed as described in 4.2.6.

6.2.7 Isolation of ACE inhibitory peptides

Isolation of ACE inhibitory peptides was conducted in a three-step purification process by subsequently subjecting samples to ultrafiltration, gel filtration and reverse phase high performance liquid chromatography (RP-HPLC). Fractions collected from each step were freeze dried. Small red bean protein hydrolysates were firstly separated into high molecular mass (MM) fraction (>5 kDa) and low MM fraction (<5 kDa) by ultrafiltration using OM005047 membranes (molecular weight cut off: 5 kDa; Pall Life Sciences, Ann Arbor, MI, USA). Subsequently, the permeate fraction (low MM fraction) having a higher ACE inhibitory activity was subjected to a HiLoad 16/60 Superdex 30 pg column

(1.6 × 60 cm; Pharmacia Co., Uppsala, Sweden). The column was pre-equilibrated with water. Freeze dried samples from the permeate fraction was dissolved in water at 20 mg/mL and filtered through a 0.45 µm membrane. 1 mL of the sample was loaded on the column and eluted using water at a flow rate of 1 mL/min. The elution was monitored at 280 nm. The fraction demonstrating the highest ACE inhibitory activity was further fractionated by using RP-HPLC with a Luna C18 reverse-phase column (21.2 × 250 mm, 5 µm particle size, 100 Å; Phenomenex, Torrance, CA, USA) connected to a Shimadzu system (Shimadzu Corporation, Tokyo, Janpan). 1 mL of the peptide solution, prepared by dispersing in water at a concentration of 5 mg/mL and filtered through 0.45 µm membrane, was injected into the column and eluted at a flow rate of 5 mL/min. The mobile phase comprised two buffers: solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). The program was started with a linear increase of solvent B from 0% to 35% in 60 min, and then maintained at 35% of B for 40 min. The elution was monitored at 220 nm.

6.2.8 Kinetics of inhibition

The inhibition kinetics was conducted as described by Barbana and Boye (2011). Briefly, ACE inhibition tests were conducted in the presence of different concentrations of HHL and peptide solution. Inhibition type was determined by plotted Lineweaver-Burk graphs of reciprocal of HA absorbance versus reciprocal of HHL concentrations. The inhibitory constant (K_i) was determined by measuring the distance between the intersection points on the *x*-axis and the zero point of the Lineweaver-Burk plot.

6.2.9 Amino acids sequence determination

Peptide samples were separated by online reverse-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analyzed by electrospray mass spectrometry (ES MS/MS). The experiments were performed with a Thermo Surveyor MS pump connected to a LTQ linear ion trap mass spectrometer

(ThermoFisher, San Jose, CA, USA) equipped with a nanoelectrospray ion source (ThermoFisher, San Jose, CA, USA). Peptide separation took place on a self-packed PicoFrit column (New Objective, Woburn, MA, USA) packed with Jupiter (Phenomenex) 5 µm particle size, 300 Å, C18, 10 cm × 0.075 mm internal diameter. Peptides were eluted with a linear gradient from 2-50% solvent B (acetonitrile, 0.1% formic acid) in 30 min, at 200 nL/min (obtained by flow-splitting). Mass spectra were acquired using a data dependent acquisition mode using Xcalibur software version 2.0. Each full scan mass spectrum (400 to 2000 m/z) was followed by collision-induced dissociation of the seven most intense ions. The dynamic exclusion (30 sec exclusion duration) function was enabled, and the relative collisional fragmentation energy was set to 35%.

All MS/MS samples were analyzed using Mascot (version 2.3.0, Matrix Science, London, UK). Mascot was set up to search the Uniref1202 database *Phaseolus vulgaris*. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 2.0 Da. Iodoacetamide derivative of cysteine was specified as a fixed modification and oxidation of methionine was specified as a variable modification. Two missed cleavage were allowed.

Scaffold (version 3.5.1, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

6.2.10 Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple comparison tests were used to determine the significant differences between means ($P < 0.05$) of ACE inhibition of different fractions from gel filtration, and IC_{50} values from fractions collected from different steps of purification using SAS Server Interface (version 2.0.3, SAS Institute, Cary, NC, USA).

6.3 Results and discussion

6.3.1 Enzymatic hydrolysis

The HPLC size exclusion chromatography profile of the small red bean protein hydrolysate is shown in Fig. 6-1. In general, the hydrolysate elicited a bell shape chromatogram with a single peak. The most abundant peptides eluted at 26.8 min, which corresponded to a MM of around 0.698 kDa. Lesser quantities of smaller and larger peptides were also obtained ranging from less than 0.244 kDa to around 7.4 kDa. In a previous study we reported that small red bean protein isolates contained a major protein with MM of 47 kDa (Rui et al., 2011). The current study, i.e. pre-heat treatment of protein isolates, and the subsequent enzymatic hydrolysis with Alcalase, papain, and in vitro GIS, clearly shows that the digestion treatment used helped to break down the large protein.

The IC_{50} value of the small red bean protein hydrolysates was determined to be 67.2±1.8 μg protein/mL (Table 6-1). This compares very well with the lower end IC_{50} values reported from digests of several other pulse proteins, such as chickpea (IC_{50}: 180 – 316 μg/mL) (Barbana & Boye, 2010; Pedroche et al., 2002; Yust et al.,2003), pea (IC_{50}: 70 – 412 μg/mL) (Barbana & Boye, 2010; Vermeirssen et al., 2005; Vermeirssen et al., 2004), and lentil (IC_{50}: 53 – 190 μg/mL) (Barbana & Boye, 2011). Lower IC_{50} value represents higher ACE inhibitory activity. Thus, the results indicate the effectiveness of the enzymatic digestion process used in the present study to release ACE inhibitory peptides from small red bean

proteins. The *in vitro* GIS digestion used in the final step of digestion in the current study was considered to be particularly important for bioavailability (Barbana & Boye, 2011), as *in vivo* ACE inhibitory activity expression requires that ACE inhibitory peptides escape from proteolytic digestion during gastrointestinal digestion, and reach the target cell with preservation of their bioactive activities. Peptides generated by *in vitro* GIS digestion are expected to be resistant to further *in vivo* gastrointestinal digestion and could, therefore, be absorbed in their intact form to deliver the potential bioactivity (Megías et al., 2004).

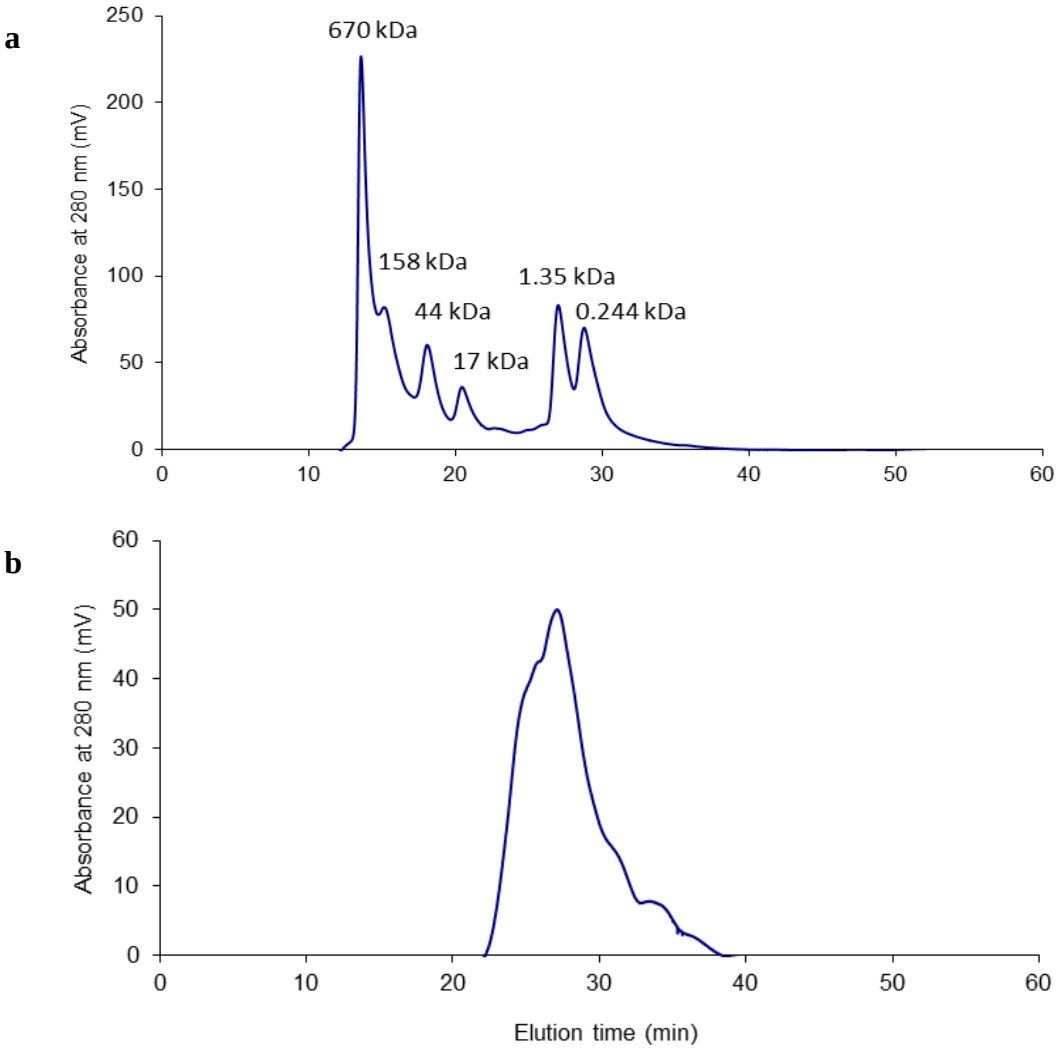


Fig. 6-1. HPLC size exclusion chromatography patterns using Biosep-SEC S2000 column of: (a) standards; (b) SPI-AP-GIS (sequential digestion of SPI (small red bean protein isolate) using AP (Alcalse/papain) followed by GIS (*in vitro* gastrointestinal digestion simulation)).

Table 6-1 Summary of the purification process and ACE inhibition of peptides generated from small red bean protein hydrolysates

Purification Step	Fraction	ACE inhibition (%)*	IC_{50} (μg protein /mL)	Purification fold
Protein hydrolysates			67.2 ± 1.8[a]	1
Ultrafiltration	Permeate	78.2 ± 2.9%[a]	50.8 ± 4.4[b]	1.3
	Retentate	68.4 ± 1.7%[b]		
Gel filtration	F5**		35.1 ± 1.8[c]	1.9
RP-HPLC	Fraction 28**		19.3 ± 1.4[d]	3.5

Data showed are means of measurements ± standard deviations; for the ACE inhibitory activity, different superscript letters within row represent significant difference ($P<0.05$)

* 25 μg of hydrolysates were used for ACE inhibition assay.

** F5 – fraction collected from gel filtration as shown in Fig. 6-2; Fraction 28 – fraction collected from preparative RP-HPLC as shown in Fig. 6-3a.

6.3.2 Purification of ACE inhibitory peptides

Several studies have reported that ACE inhibitory peptides are mainly associated with peptides of low molecular weights (Kuba et al., 2005; Vermeirssen et al., 2005; Wu & Ding, 2002; Zhu et al., 2010). Therefore in the fractionation and purification steps, small red bean protein hydrolysates were firstly passed through a membrane with molecular weight cut off (MWCO) of 5 kDa to remove the large peptides. As expected, the permeate fraction which represented low molecular weight peptides was determined to have significantly higher ($P<0.05$) ACE inhibitory activity, i.e. 78.2±2.9% (for 25 ug of hydrolysates) than that of the retentate fraction (68.4±1.7%, for 25 ug of hydrolysates). This fraction was, therefore, collected for further purification. An improvement of IC_{50} value (50.8±4.4 μg protein/mL) was obtained for the permeate fraction compared to that of the protein hydrolysate (67.2±1.8 μg protein/mL) (Table 6-1).

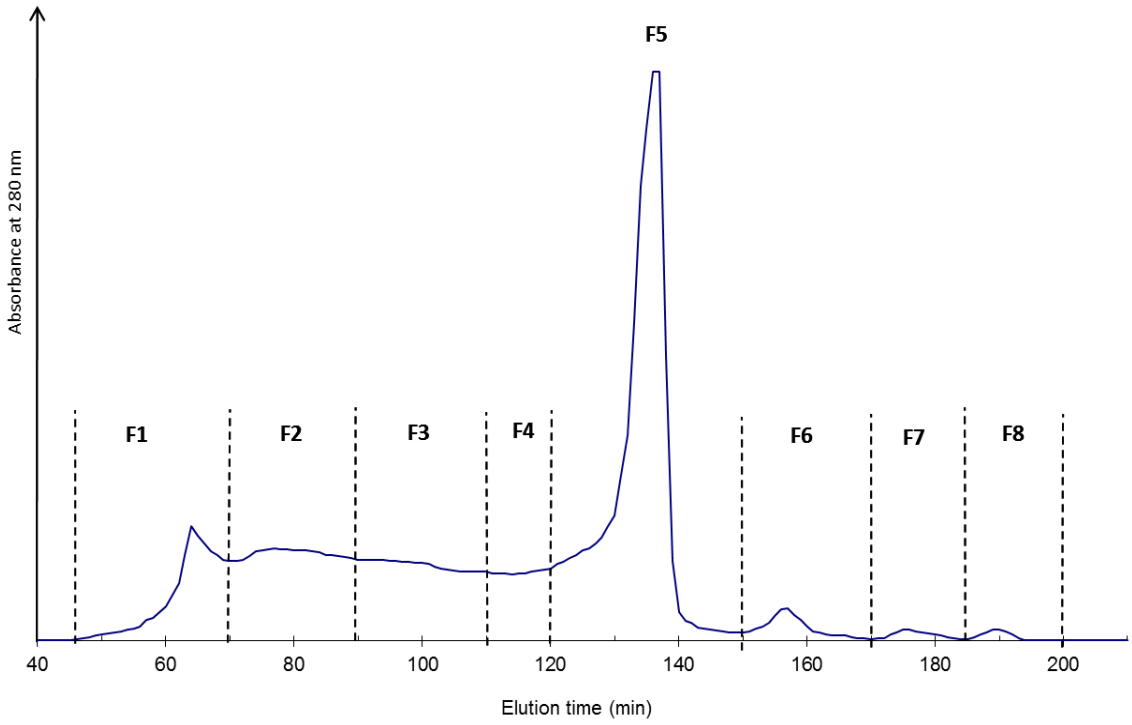


Fig. 6-2. Gel filtration chromatographic profile using HiLoad 16/60 Superdex 30 pg column of permeate fractions obtained by ultrafiltration. Fractions 1-8 were collected as shown in the figure and lyophilized for ACE inhibitory assays.

Table 6-2 ACE inhibition of fractions separated by gel filtration

Fraction	Elution time (min)	ACE inhibition (%)*	Yield (%)**
F1	46-70	60.9± 3.8%[a]	10.7
F2	71-90	1.0± 0.9%[c]	18.2
F3	91-110	6.7± 5.0%[c]	15.7
F4	111-120	47.2± 2.1%[b]	7.1
F5	121-150	59.6± 0.5%[a]	44.4
F8	171-184	7.7± 4.7%[c]	0.6

Data showed are means of measurements ± standard deviations; for the ACE inhibitory activity, different superscript letters within row represent significant difference (P<0.05).
* 10 µg of hydrolysates were used for ACE inhibition assay.
** Yield was calculated based on weight of freeze dried samples.

IC_{50} value improved three times for soybean protein hydrolysates after membrane filtration using a MWCO of 5 kDa (Wu & Ding, 2002). Similar improvement was reported for whey protein hydrolysates ultrafiltered with a 3 kDa MWCO membrane (Vermeirssen et al., 2005). Although the improvement in the bean sample (1.3 times, based on IC_{50} values) was not as pronounced as some of these previous reports which used a similar ultrafiltration process to separate ACE inhibitory peptides, the increase is similar to that observed for pea digests (Vermeirssen et al., 2005), which has a more similar protein profile to the bean used in this study (i.e., also considered a pulse legume).

Ultrafiltration is a simple, fast and reliable way for enrichment of bioactive peptides, and it has also been acknowledged as a technic suitable for industrial scale applications (Vermeirssen et al., 2005; Zhu et al., 2010). Our results confirm that it can be used as a processing technique to generate a crude pool of bioactive peptides with higher ACE inhibitory activity compared to the whole protein hydrolysate.

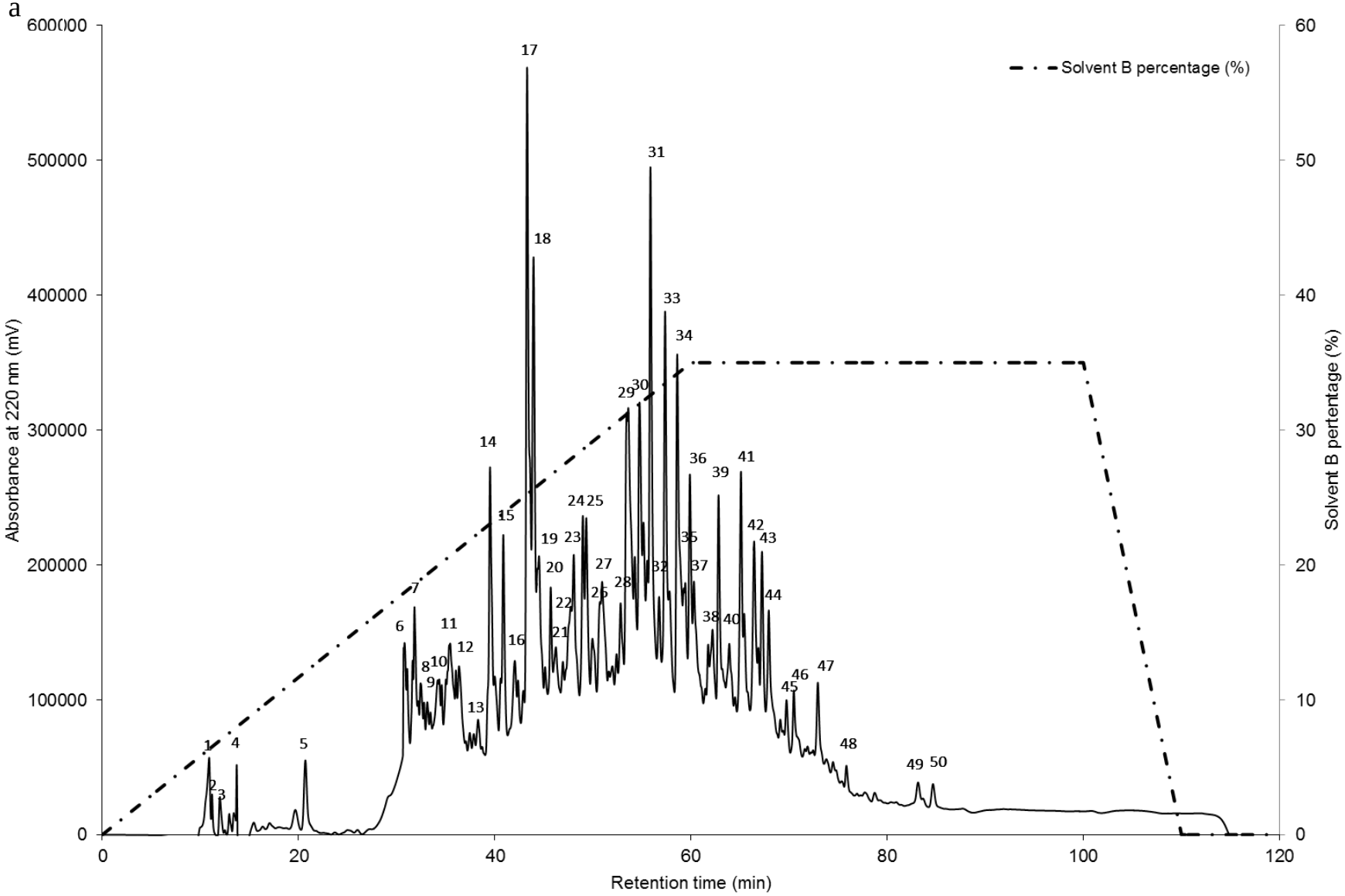
The permeate fraction was freeze dried and loaded to a HiLoad 16/60 Superdex 30 pg column for further purification. The chromatogram presented in Fig. 6-2 showed most peptides eluting between 46 min to 150 min. The permeate fraction comprised a minor fraction which eluted early with a peak at elution time of 64 min, which indicated that it contained a group of larger peptides, and a major fraction which peaked at 136-137 min, indicating that it contained a group of smaller peptides. As shown in Fig. 6-2, eight fractions (F1-F8) were collected. All fractions exerted ACE inhibitory activity except F6 and F7. The highest ACE inhibitory activity ($P<0.05$) was obtained for fractions F1 and F5, which demonstrated values of 60.9±3.8% and 59.6±0.5%, respectively (based on 10 µg hydrolysates) (Table 6-2). No significant differences ($P>0.05$) were observed between them. As indicated above, high ACE inhibitory activity is normally associated with peptides of low molecular mass. Occasionally, for certain protein hydrolysate the reverse phenomenon has been observed (Megías

et al., 2004). The present results, therefore, suggested that there may be some large peptides in small red bean hydrolysates which elicited comparable ACE inhibitory activity to the smaller ones. With the consideration that lower molecular mass peptides have a greater likelihood of crossing the intestinal barrier to deliver their bioactivity (Vermeirssen et al., 2005), coupled with the higher yield of the F5 fraction (Table 6-2), the F5 fraction was chosen for further assays. IC_{50} value of the F5 fraction improved and was determined to be 35.1±1.8 μg protein/mL.

The RP-HPLC profile of the F5 fraction is shown in Fig. 6-3a. The chromatogram contained several peaks suggesting that the F5 fraction was composed of a mixture of peptides. Most of the peaks eluted after the acetonitrile concentration reached 17.5%. Fifty fractions were separated as shown in Fig. 6-3a and they were collected for ACE inhibitory assays.

Most of the fractions elicited ACE inhibitory activity (Fig. 6-3b). Fraction 28 which eluted at around 30% of acetonitrile demonstrated the most potent ACE inhibitory activity of 77% (based on 10 μg hydrolysates). Additionally, several active fractions inhibiting more than 50% of ACE activity were observed which mainly eluted in two groups, i.e., peak 17 to 23, and peak 35 to 41. Other studies have reported that fractions with high ACE inhibitory activity are normally obtained at the middle or end phase of RP-HPLC chromatogram which correspond to peptides with lower molecular mass and which have greater amounts of hydrophobic amino acids (Mallikarjun Gouda et al., 2006; Megías et al., 2004; Yust et al., 2003). Our results are in accordance with these findings. Fraction 28 demonstrated an IC_{50} of 19.3±1.4 μg protein/mL and was collected for further characterization studies.

a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Retention time (min)
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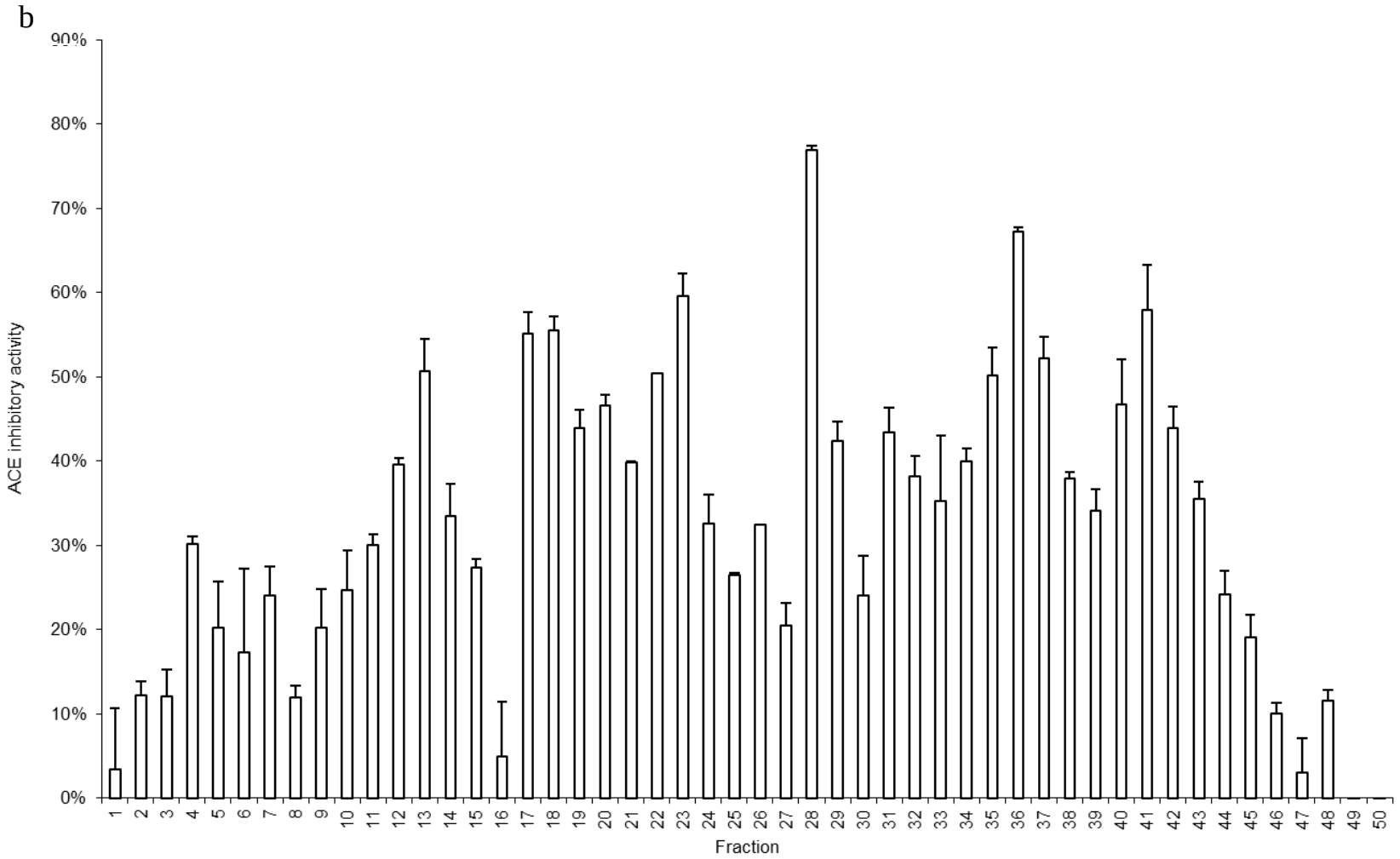

Fig. 6-3. (a) Preparative RP-HPLC profiles using Luna C18 reverse-phase column of active fraction, F5, obtained from gel filtration. Fractions 1-50 were collected as shown in the figure and lyophilized for ACE inhibitory assays. (b) ACE inhibitory activity of fractions collected from the preparative RP-HPLC step. 10 µg of hydrolysates were used for ACE inhibitory tests.

6.3.3 Kinetics of inhibition

Inhibition kinetics of Fraction 28 collected from the previous RP-HPLC was determined by Lineweaver-Burk plot. As shown in Fig. 6-4, increasing the peptide concentration generated lines with coinciding intercept at *y*-axis, which suggested that the inhibitors from fraction 28 were of the competitive type. This indicates that the inhibitory peptides acted through binding of the active site of the ACE and, thus, blocked the enzyme from interacting with substrate. Many of the ACE inhibitory peptides from food sources reported in the literature were also found to be competitive inhibitors (e.g., peptides derived from soybean Alcalase hydrolysates (Wu & Ding, 2002), glycincin digests hydrolyzed by protease P (Mallikarjun Gouda et al., 2006), and chickpea protein Alcalase/Flavourzyme hydrolysates (Pedroche et al., 2002)), although non-competitive inhibitory patterns (Barbana & Boye, 2011) and uncompetitive inhibitory patterns have been reported (Pedroche et al., 2002).

The inhibition constant (K_i) value of Fraction 28 was also determined from the Lineweaver-Burk plot and is shown in Fig. 6-4. The K_i value indicates the affinity between an inhibitor and an enzyme. In general, lower K_i values indicate higher affinity. The K_i value obtained for Fraction 28 was 11.6±1.7 µg protein/mL. This was at least 10 times lower compared to the previous K_i values reported for crude ACE inhibitory peptides from lentil hydrolysates, i.e. 0.16–0.46 mg/mL (Barbana & Boye, 2011), which indicates a more potent inhibitory activity.

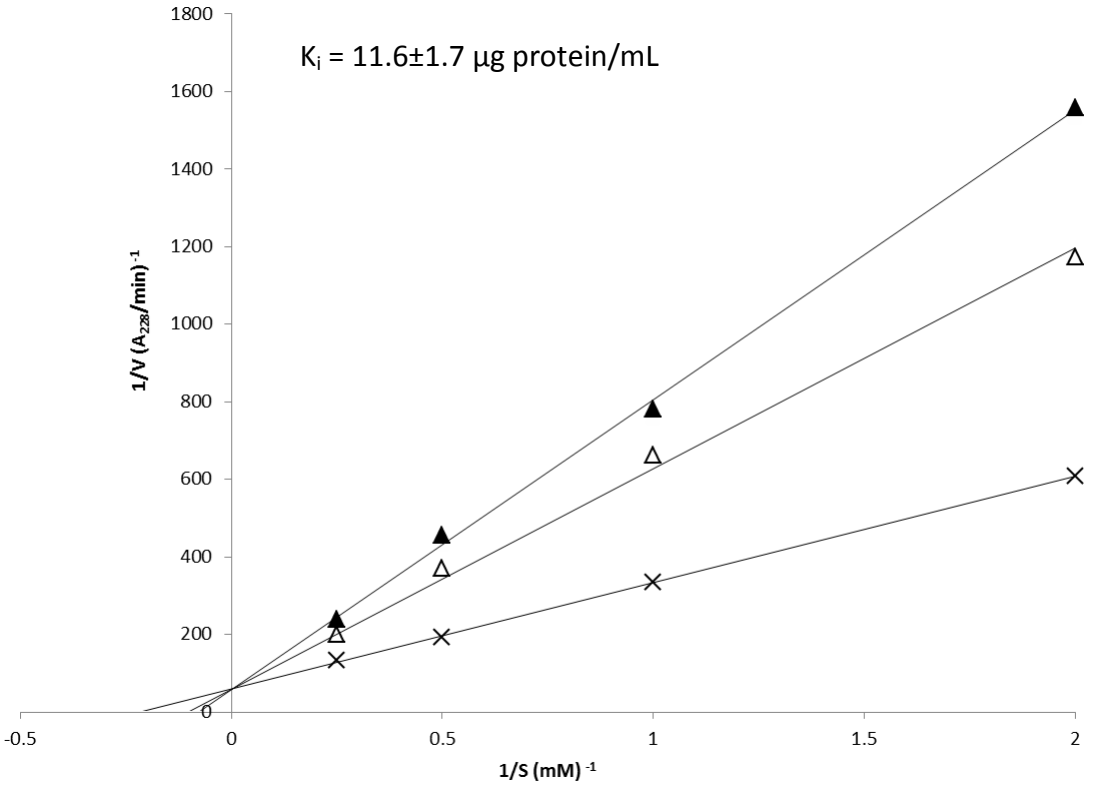

Fig. 6-4. Lineweaver-Burk plot for the inhibition of ACE by Fraction 28 collected from the preparative RP-HPLC step. (-×-) without inhibitor; (-Δ-) 11.3 µg protein/mL inhibitory peptides; (-▲-) 22.6 µg protein/mL inhibitory peptides. Each point represents the average of two replicates. Inhibition constants (K_i) are indicated on the graph and are shown as means of two replicates ± standard deviations.

6.3.4 Mass spectrometric characterization of selected peptide fraction

Peptide sequencing using tandem mass spectrometry showed that Fraction 28 was not composed of a single peptide but several peptides. A screening of the results demonstrated the presence of an octapeptide PVNNPQIH, which appeared at a frequency three times higher, compared to the peptide with the second highest concentration. This peptide was likely a predominant fraction in the peptide mixture which may have contributed to the potent ACE inhibitory activity of Fraction 28. A search of the intact protein sequence showed that the octapeptide PVNNPQIH originated from the major storage protein phaseolin, (Fig.

6-5). The peptide was synthesized and demonstrated an IC_{50} value of 206.7±3.9 µM. This peptide, to the best of our knowledge, is a newly identified ACE inhibitory peptide originating from dry bean protein.

The relationship between structural properties and ACE inhibitory activity remains obscure (Fang et al., 2008). High proline content has been reported to be a typical characteristic of peptides with high ACE inhibitory activities (Meisel, 1997). The peptide identified in the current study contained two prolines which is in agreement with these previous findings. Additionally, the PQ domain observed from the current peptide shares a similarity to several previous reports, such as KVLPVPQ from β-casein (Maeno et al., 1996), YKVPQL from αs1-casein (Maeno et al., 1996), FALPQY from bovine αs2-casein (Tauzin et al., 2002), and FVNPQAGS from sunflower helianthinin (Megías et al., 2004). The consistent appearance of this domain in ACE inhibitory peptides may imply some structure-activity relationship between the inhibitor and enzyme.

MVRARVPLML LGILFLASLS ASFATSLREE EESQDNPFYF NSDNSWNTLF KNQYGHIRVL
QRFDQQSKRL QNLEDYRLVE FRSKPETLLL PQQADAELLL VVRSGSAILV LVKPKKRREY
FFLTSDNPIF SKHQKIPAGT IFYLVNPDPK EDLRIIQLAM **PVNNPQIH**DF FLSSTEAQQS
YLQEFSKHIL EASFNSKFEE INRVLFEEEG QQEGVIVNID SEQIKELSKH AKSSSRKSLS
KQDNTIGNEF GNLTERTDNS LNVLISSIEM KEGALFVPHY YSKAIVILVV NEGEAHVELV
GPKGNKETLE YESYRAELSK DDVFVIPPAAY PVAIKATSNV NFTGFGINAN NNNRNLLAGK
TDNVISSIGR ALDGKDVLGL TFSGSGDEVM KLMNKQSGSY FVDAHHHQQE QQKGRKGAFV
Y

Fig. 6-5. Sequence of the two most prominent peptides identified by tandem mass spectrometry from Preparative RP-HPLC Fraction 28 and its location in phaseolin.

6.4 Conclusions

This study was the first to purify and characterize ACE inhibitory peptides from small red bean (*Phaseolus vulgaris*) protein hydrolysates. A potent ACE inhibitory fraction was obtained using a three step purification process which showed competitive inhibition. The results clearly demonstrate that small red

bean protein could be a good source for generating ACE inhibitory peptides. Furthermore, the study provides detailed theoretical knowledge for the purification of ACE inhibitory peptides from dry beans which could be expanded to other pulse legumes. Further studies on the *in vivo* behaviour of the potent fractions selected are required. As sample size is frequently a limitation for such in vivo studies, optimisation and scale up of the purification steps described should allow larger amounts of the purified fractions to be generated for future animal or human studies.

Chapter 7: General Conclusions and Recommendations

7.1 Conclusions

In this study, antihypertensive properties of nine dry bean varieties that are largely grown in Canada were investigated. The following summarizes the important findings of this study.

Our results showed that all investigated dry beans had similar protein profiles. All varieties contained the major protein phaseolin with estimated molecular mass (MM) of 47 kDa.

Phaseolin type, however, differed for cranberry and light red kidney beans which were categorized as T-type phaseolin, whereas that of the other seven varieties was classified as S-type phaseolin.

Two varieties, i.e., cranberry and light red kidney beans had significantly lower ($P<0.05$) thermal stabilities than the other seven varieties.

Black bean protein isolate showed a distinctive protein profiles which was missing some proteins in the MM range of 60-97 kDa. It also had larger amounts of high MM proteins. Navy, great northern and black bean protein isolates also had different profiles especially in the low MM region.

FTIR studies showed that the nine dry bean varieties had similar secondary structure with predominantly β-sheet structure. Increasing the temperature from 25 °C to 60 °C led to a reduction of β-sheet structure content and an increase in the intensity of bands representing random coil structures. Furthermore, higher temperature (i.e, from 60 °C to 95 °C) induced the formation of intermolecular β-sheets.

All nine varieties of dry bean hydrolysates demonstrated *in vitro* ACE inhibitory activities after hydrolysis using either trypsin or *in vitro* gastrointestinal simulation (GIS).

Despite large amounts of phaseolin which remained intact after *in vitro* GIS digestions for all investigated varieties, the degree of hydrolysis (DH) values for

the GIS samples were significantly higher ($P<0.05$) than that of samples digested with trypsin alone. ACE inhibitory activities for all nine dry bean hydrolysates were similarly affected.

Among the nine varieties, small red bean protein hydrolysate had significantly higher ($P<0.05$) ACE inhibitory activity t (IC_{50}s: 170 μg protein/mL for tryptic digestion and 118 μg protein/mL for *in vitro* GIS), followed by navy bean (IC_{50}s: 200 μg protein/mL for tryptic digestion and 137 μg protein/mL for *in vitro* GIS).

Cranberry and light red kidney bean hydrolysates, which after both tryptic and *in vitro* GIS digestions, showed higher DH values and discrepant peptides profiles compared to other seven varieties, elicited average ACE inhibitory activities.

Black bean protein hydrolysates exerted significantly higher ($P<0.001$) ACE inhibitory activity when subjected to *in vitro* GIS digestion (IC_{50}: 150 μg protein /mL) compared to tryptic digestion only (IC_{50}: 406 μg protein/mL).

Pre-heat treatment (100 °C, 15 min) of the protein isolates facilitated the degradation of phaseolin during subsequent enzymatic treatments for the three investigated varieties, i.e. navy, black and small red bean, but induced the formation of protein aggregates for black bean (which were resistant to further hydrolysis). DH values improved after pre-heat treatment for both navy and small red bean, as did their ACE inhibitory activities. However, decreased DH values were obtained for black bean, and the ACE inhibitory activity was not significantly different ($P>0.05$) for samples with/without pre-heat treatment.

ACE inhibitory activities of hydrolysates produced using a dual-enzyme combination treatment, i.e. Alcalase/Flavourzyme and Alcalase/papain showed that the latter combination was more effective in generating ACE inhibitory peptides from navy, black and small red beans, despite data showing that the digestion of papain presented lower DH values compared to Flavourzyme.

The highest ACE inhibitory activities were obtained for samples digested for 100 min, 95 min, and 95 min with Alcalase/papain digestion. IC_{50} values for these samples were 68 µg protein/mL, 83 µg protein/mL, and 78 µg protein/mL for navy, black and small red bean protein hydrolysates, respectively. Improved ACE inhibitory activities were obtained when these hydrolysates were further subjected to *in vitro* GIS digestions in the order of small red bean > navy bean >black bean.

Small red bean hydrolysate, digested by Alcalase/papain and subsequently *in vitro* GIS digestion, followed by a three-step purification process, including ultrafiltration, gel filtration and preparative reverse phase high performance chromatography (RP-HPLC) yielded a fraction with a high IC_{50} value of 19.3 µg protein/mL.

Enzymatic kinetic studies showed the fraction to be a competitive inhibitor with K_i of 11.6 µg protein/mL, and comprised mostly of peptide with amino acid sequences PVNNPQIH.

Overall, the results from the current study expand the scientific knowledge of the physicochemical properties of the nine dry bean (*Phaseolus vulgaris*) varieties studied and provides new knowledge of their biochemical properties. The comparative studies of the *in vitro* ACE inhibitory activities, protein profiles, thermal properties and secondary structures are the first to be reported in the literature to the best of our knowledge. Results from this work provide a solid base for further research on the preparation of ACE inhibitory fractions from pulses and should be useful for selecting conditions for the preparation of larger quantities of material for *in vivo* studies or in the preparation of hydrolysates and functional ingredients for novel foods targeting hypertension.

7.2 Recommendations for future studies

This study has provided several important findings regarding the *in vitro* ACE inhibitory activities of dry bean (*Phaseolus vulgaris*) proteins. Further studies

that could be useful in bringing dry bean protein hydrolysates as antihypertensive ingredient to our dining table include the following:

a) Study of the *in vivo* antihypertensive properties of selected bean protein hydrolysates (e.g., small red bean protein hydrolysates produced by Alcalase/papain), or partially purified bioactive fraction (e.g., permeate fraction collected from ultrafiltration).
b) Study of the stability of the dry bean protein hydrolysates when exposed to treatments likely to be encountered during food processing (e.g., thermal treatment, high pressure treatment, alteration of pH, etc.)
c) Study of the sensory properties of dry bean protein hydrolysates as peptides may have a bitter taste which might alter food sensory properties. In this regard, several techniques such as masking and enzymatic treatment may be considered.
d) Optimization and scale up of the preparation and fractionation processes developed to increase production scale.

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