PEANUT (*Arachis hypogeae* L.) AND COWPEA (*Vigna unguiculat* L.) AS SOURCES OF ANGIOTENSIN I CONVERTING ENZYME INHIBITORY PEPTIDES

by

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(Under the Direction of Robert Dixon Phillips)

ABSTRACT

A microplate kinetic assay was used to examine the degradtion of substrate N-[3-(2-furyl) acryloyl]-L-phenylalanylglycylglycine (FAPGG) by angiotensin I converting enzyme (ACE) to furylacryloylphenylalanine (FAP) and glycylglycine (GG). The slopes of resulting kinetic curves for the first 15 min were used to calculate ACE inhibition. For food protein hydrolysates, ACE inhibition began to plateau after peanut was hydrolyzed for 2.5 h and cowpea for 1 h. A Box-Behnken statistical screening experiment with three levels was performed to evaluate the effects of hydrolysis factors pH, temperature and enzyme to substrate (E/S) ratio on ACE inhibitory activities of Alcalase hydrolysates of peanut and cowpea flours using response surface methodology (RSM). The factor pH did not show significant influence, whereas hydrolysis temperature and E/S ratio had significant effects on ACE inhibitory activities over their ranges studied. Response surface modelings and optimal hydrolysis conditions were determined. Peanut and cowpea hydrolysates obtained after 6 h of digestion under central point conditions by Alcalase were used to isolate ACE inhibitory peptides. After a series of purifications by membrane separation, primary and secondary reverse-phase high performance liquid

chromatographies, the active peptides were found to be Lys-Ala-Phe-Arg for peanut and Phe-Phe for cowpea sequenced by an matrix-assisted laser desorption and ionization (MALDI) tandem TOF-TOF (time-of-flight) mass spectrometer. Quantitative structure-activity relationship (QSAR) of ACE inhibitory dipeptides was studied by partial least square (PLS) regression based on four sets of amino acid descriptor scales. The activies of collected dipeptides including the isolated dipeptide FF from Alcalase hydrolysate of cowpea were predicted. A potent ACE inhibitory dipeptide was proposed to have a large and hydrophobic amino acid such as Trp and Phe at the C-terminus and a nonpolar amino acid such as Val, Leu and Ile or possibly a positively charged amino acid such as Arg and Lys at the N-terminus.

INDEX WORDS: Peanut and cowpea hydrolysates, Angiotensin I converting enzyme, Microplate reader, FAPGG, Response surface methodology, Reverse-phase high performance liquid chromatography, Mass spectrometry, Quantitative structure-activity relationship, Partial least square, Amino acid descriptors, ACE inhibitory peptides.

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DEDICATION

In memory of my mother, Guilin Nie. She gave me a mother's love, discipline, and direction. I miss her forever.

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CHAPTER 1

INTRODUCTION

Hypertension is one of the most common worldwide diseases that afflict humans. Angiotensin I converting enzyme (ACE) catalyzes the formation of vasoconstrictor, angiotensin II and the inactivation of vasodilator, bradykinin. The influences of ACE on blood pressure make it an ideal target clinically and nutritionally in the treatment of hypertension. Although peptide analogues as clinical drugs have shown their usefulness for first-line therapy of hypertension, they are not entirely without side-effects. Also, new findings demonstrated that two halves of ACE bear little structure similarity to carboxypeptidase A on which the initial drug development of ACE inhibitors was based.

ACE inhibitory peptides from frequently consumed foods are attracting considerable interest because they are more natural and safer when compared with ACE inhibitory drugs. As neutraceuticals added to functional foods, these food-derived ACE inhibitory peptides could be applied in the prevention of hypertension and as initial treatment in mildly hypertensive individuals. ACE inhibitory peptides have been isolated from various animal and plant food sources. Among the plant food sources, soybean and related products have been the most widely studied. Peanut (*Arachis hypogeae* L.) and cowpea (*Vigna unguiculat* L.), two other important grain legumes, are both rich sources of protein. The values of peanut and cowpea could be increased if processed to contain abundant ACE inhibitory peptides.

The aim of this reseach is therefore to produce, isolate and identify ACE inhibitory peptides from peanut and cowpea proteins. The assay for the determination of ACE inhibition needs to be established according to new laboratory conditions; hydrolysis parameters affect peptide compositions and therefore ACE inhibitory activities of hydrolysates; purification is achieved by membrane and chromatographic separation techniques and peptides is sequenced by mass spectometers; ACE inhibitory activity of a peptide is a function of its compositional amino acids and, therefore, the relationship between structure and activity is established.

Chapter 2 in this study is the literature review, which primarily covers three core topics: peanut and cowpea proteins, ACE, and plant food-derived ACE inhibitory peptides. Chapter 3 looks at the kinetic progress of ACE-catalyzed reactions and establishes an assay for determining ACE inhibition. Chapter 4 describes the effects of hydrolysis factors on ACE inhibitory activities of Alcalase hydrolysates of peanut and cowpea flours. Chapter 5 describes the purification and sequence of ACE inhibitory peptides. Chapter 6 looks at the quantitative structure-activity relationship of ACE inhibitory dipeptides.

CHAPTER 2

LITERATURE REVIEW

Content and amino acid profiles of peanut and cowpea proteins

Peanut (*Arachis hypogeae* L.) and cowpea (*Vigna unguiculat* L.), two important grain legumes, are both rich sources of protein. Crude protein content of whole seed peanuts ranges between 22 and 30% (Pancholy *et al*., 1978). Some wild species (Grosso *et al*., 2000), genetically modified cultivars (Jonnala *et al*., 2005; Ng *et al*., 2008), and hydroponically grown peanut seeds (Wu *et al*., 1997) also have the variable protein contents within the range. In cowpea cultivars, protein content ranges from 20-34.2% with a mean of 24% (Ofuya and Akhidue, 2005).

Amino acids glutamine/glutamic acid and asparagine/aspartic acid are the most abundant in both legume proteins (Pancholy *et al*., 1978; Khalil and Chughtai, 1983a; Andersen *et al*., 1998) and thus make them typically acidic. Peanut protein is a good source of arginine and histidine but contains relatively low amount of methionine/cystine. When compared to chickpea, lentil and green pea, cowpea seed protein has higher essential amino acid content (Iqbal *et al*., 2006). It is relatively rich in lysine but deficient in sulfur amino acids (methionine/cystine) and tryptophan (Chan and Phillips, 1994; Iqbal *et al*., 2006; Leticia *et al*., 2007). Several wild cowpea species have very high content of cystine, which suggests that the cystine content of cultivated cowpeas could be increased by interspecific breeding (Marconi *et al*., 1997). Variations in amino acid profiles may be due to genetic and environmental factors. The sample preparations and measuring

methods for protein content and amino acid composition may also cause difference in reported results.

Processing into peanut and cowpea protein products

A series of extractions with organic solvents can remove fat in peanut and thus efficiently increase the protein content. Defatted peanut meals have protein content ranging between 40% and 55%. Due to low fat content of cowpea, defatting processing plays little role in increasing protein content. Protein concentrates result from removing non-protein constituents, mainly soluble minerals, carbohydrates, low molecular weight nitrogen compounds and antinutritive factors from full fat or, more usually, from defatted meals at neutral or in acid medium. The production of isolates consists of an aqueous solubilization of protein and carbohydrates at neutral or alkaline pH and the selective recovery of the solubilized protein by adjusting pH to the isoelectric point where solubility is minimized, separation and, optionally, washing and neutralization before drying (Moure *et al*., 2006). Generally, isolates have higher protein content than concentrates and defatted meals. The sample preparation and extraction protocol affect the protein extraction efficiency. The optimum recovery of peanut protein can be achieved at alkaline pH. Poms *et al*. (2004) reported higher protein extraction efficiency from the raw peanut than from roasted peanuts and found that elevated roasting temperature resulted in a significant decrease in protein extraction efficiency. Yu *et al*. (2007), however, found the protein isolate developed from defatted roasted peanut flour had higher protein content than from the defatted raw flour. The authors explained the

higher fat content led to the decreased protein extraction efficiency from defatted raw flour due to the formation of an emulsion in conjunction with protein during extraction.

Depending on the type and amount of extraction medium used, different quantities of protein fractions with varying solubility properties can be prepared from peanut and cowpea. Peanut seed extracts contain albumins (water soluble), globulins (salt or buffer solution-soluble - arachin and conarachin), and a number of other protein components distinguishable by chromatographic and standard polyacrylamide gel electrophoretic techniques (Cherry, 1990). Globulins are reported to be the major cowpea seed protein, ranging between 48.2 and 90%. The remaining soluble protein fractions are albumins, glutelins (soluble in dilute acids and bases) and prolamins (soluble in aqueous alcohol) with varying contents (Chan and Phillips, 1994; Nugdallah and El Tinay, 1997). Each fraction can be further divided into subunits having different molecular masses. Heating peanut and cowpea seed proteins significantly decreases the level of albumin and globulin fractions with concomitant increase in apparent glutelin fraction (Patil *et al*., 1991; Nugdallah and El Tinay, 1997).

Functional properties of peanut and cowpea proteins

Functional property is any property of a substance, besides nutritional, that affects its utilization. The functional properties that influence the role of proteins in food applications are color, flavor, texturization, solubility, viscosity, adhesion or cohesion, gelation, coagulation, aeration or foamability, water and oil absorption and emulsification. Of these, solubility, foamability and emulsification are popular to study because they form the basis of many food systems. Factors that influence solubility

properties, including protein composition and structure, methods and conditions of extraction, processing and storage, or treatments used to modify protein character, influence protein foamability and emulsification (Cherry, 1990).

Protein solubility is influenced by various factors, such as hydrophobic residues, charges, electrostatic repulsion, ionic hydration, pH, denaturation, salting-in and salting-out (Moure *et al*., 2006). In the pH-solubility profiles, peanut and cowpea proteins both exhibit a u-shaped curve in which the minimum solubility of proteins is observed at pH 3.5-5.5 and maximum solubility at alkaline pH (Park *et al*., 1990; Horax *et al*., 2004; Ragab *et al*., 2004; Yu *et al*., 2007; Kain and Chen, 2008). Mild roasting treatments to peanut kernels are reported to increase protein solubility (Damame *et al*., 1990; Patil *et al*., 1993), while severe heating is known to decrease it (Basha and Young, 1985; Chiou and Tsai, 1989; Patil *et al*., 1991). This decrease can be explained by the effect of heating which increases surface hydrophobicity of protein due to unfolding of molecules upon heat and molecular size through hydrophobic interactions and disulfide formation (Yu *et al*., 2007). In the progressive roasting, protein solubility in the defatted peanut flour is reduced more quickly than in full-fat peanut flour, presumably due to its greater surface area (Kopper *et al*., 2005). Yu *et al*. (2007) found fermentation significantly increased protein solubility in defatted roasted peanut flour at pH 3.0-10 but decreased protein solubility of defatted raw peanut flour at pH 6-10. Ahmed and Schmidt (1979) reported that spray- and freeze-dried peanut protein isolates had higher solubility than drum-dried isolate. Peroxide (30% H_2O_2) exposure can induce the conformational transition of arachin, the major protein of peanut, and increase its solubility to 8.5-fold (Jacks *et al*.,

1983). Gamma irradiation (Abu *et al*., 2005; Abu *et al*., 2006) and micronization (Mwangwela *et al*., 2007) significantly affect the solubility of cowpea protein.

Emulsifiers or foaming agents decrease the interfacial tension and facilitate formation of stable oil-water and air-water interfaces (Moure *et al*., 2006). The ability of proteins to act as emulsifiers varies with the molecular properties of proteins, the main factors affecting the properties of emulsions being molar mass, hydrophobicity, conformation stability, charge and physico-chemical factors such as pH, ionic strength and temperature (Kinsella, 1976). Good foaming proteins must rapidly adsorb at the air-water interface during whipping and bubbling, undergo rapid conformational change and rearrangement at the interface, and form a viscoelastic cohesive film through intermolecular interactions (Moure *et al*., 2006). After applying shear stress forces or ultrasound, the emulsifying capacity and stability are measured and expressed based directly on the emulsified oil (Sathe and Salunkhe, 1981) or indirectly on spectrophotometric absorbance of emulsion (Pearce and Kinsella, 1978). Foaming capacity and stability are calculated based directly on the volume change after whipping or aeration of a protein solution or indirectly on conductivity (Kato *et al*., 1983). Highly insoluble proteins are not good emulsifiers and can generate coalescence (Moure *et al*., 2006). Peanut concentrates and isolates produce better emulsions even though some of them have lower protein solubility than certain flours. This is probably due to the high concentration of protein, especially the major globulin, arachin, in the concentrates and isolates, which is thought to contribute greatly to the functionality of peanut products (Cherry, 1990). The extremely high emulsifying capacity of peanut protein concentrate/isolate makes it a good candidate for food formulations requiring high

emulsifying capacities such as salad dressing and creamy soup (Yu *et al*., 2007). Peanut concentrates and isolates having the higher percentage of soluble protein, are also shown to have excellent foaming properties (Cherry, 1990). The impact of roasting on emulsifying and foaming capacities of peanut protein depends on the roasting time and temperature (Patil *et al*., 1993; Yu *et al*., 2007; Kain and Chen, 2008), while fermentation and spray drying increase the two properties (Yu *et al*., 2007). The emulsifying and foaming capacities and stabilities of cowpea protein are pH-dependent and alkaline pH improves these properties more than acidic pH. The profiles of emulsifying and foaming capacities against pH for cowpea protein are more or less similar to that of its solubility against pH (Ragab *et al*., 2004). The emulsifying and foaming properties of cowpea protein are also affected by γ-irradiation (Abu *et al*., 2005; Abu *et al*., 2006). Micronization of cowpea by a short time high temperature process significantly reduces the foaming capacity of cowpea protein due to extensive protein denaturation (Mwangwela *et al*., 2007). It has been suggested that foaming properties are negatively related to protein denaturation (Yasumatsu *et al*., 1972). Partially purified cowpea globulin treated with microbial calcium-independent transglutaminase shows the reduced foaming properties because the protein crossing-linking may lead to gradual loss of flexibility and the protein's ability to unfold at the water air interface (Aluko and Yada, 1999).

Nutritional qualities of peanut and cowpea proteins

Protein quality is a function of its essential amino acid composition, digestibility and bioavailability of amino acids (FAO/WHO, 1990). Peanut and cowpea proteins are

both incomplete proteins with one or more essential amino acids deficient. The standardized rat fecal balance method is recommended for predicting digestibility of protein by humans. However, the bacterial modification in the large intestine of nitrogen excretion may result in overestimation of digestibility of protein and amino acids, particularly in poorly digestible products (Gilani *et al*., 2005). The true fecal protein digestibility values of peanut butter and peanut flour are 95% and 93%, respectively, which is comparable to animal protein digestibility. The whole seed peanut and cowpea have lower protein digestibility values of 87% and 79%, respectively (Department of Health, South Africa, 2007). The presence of less-digestible protein fractions, insoluble fiber, and antinutritional factors may be responsible for the relatively low digestibility (Gilani *et al*., 2005). Moderate heating can promote digestibility by promoting breakdown of peptide crossing-linkages, decrease or inactivation of antinutritional factors such as protease inhibitors, amylase inhibitors, hemagglutinin, HCN and phytic and tannic acids (Umoren *et al*., 1997). Strong heating/alkaline processing of protein products can yield Maillard compounds, oxidized forms of sulfur amino acids (such as methionine sulfoxide, methionine sulfone, and cysteic acid), D-amino acids, and cross-linked forms of amino acids like isopeptides, lanthionine and lysinoalanine, which significantly reduces the protein digestibility and amino acid bioavailability with lysine being the most sensitive amino acid to nutritive damage (Papadopoulos, 1989; Gilani *et al*., 2005).

Numerous methods exist to determine protein quality. For many years the standard to determine protein quality was the protein efficiency ratio, or PER. This technique requires feeding rats a test protein and then measuring the weight gain in grams per gram of protein consumed. The standard protein, casein, has a PER of 2.5. Any

protein with a PER greater than 2.5 is regarded as an excellent quality protein (Hoffman and Falvo, 2004). Rats grow very fast and have a very high requirement for methionine. So this method overestimates the quality of animal proteins and underestimates the quality of legume proteins which are generally limiting in methionine. The raw and roasted peanut pastes have PER values 1.81 and 1.50, respectively, with roasting processing changing the equally limiting amino acid sequence in raw peanut protein into the sequence lysine, threonine and methionine (Mcosker, 1961) According to Jenkins and Mitchell (1989), the defatted peanut protein has PER value 1.69. Miller and Young (1977) found the growth of rats fed diets containing 16.7 and 20% peanut protein was essentially equivalent to that of animals fed 12 to 24% casein protein, while with 13.3% peanut protein in the diet, methionine, lysine, and threonine were equally limiting in the peanut meal as measured by rat growth and PER of amino acid supplemented diets. PER values of raw cowpea protein range from 1.34 to 1.84, depending on the varieties. Autoclaving processing of cowpeas significantly increase PER values to 1.94-2.56 (Umoren *et al*., 1997). Irradiation significantly enhances PER values in a dose-dependent manner (El-Niely, 2006). PER value of sorghum flour when supplemented with peanut flour and cowpea soy flour is 2.37 comparable with casein protein and essential amino acid patterns comparable with FAO values (Okeiyi and Futrell, 1983).

Two biological methods, biological value (BV) and net protein utilization (NPU), both measure the same parameter of nitrogen retention, however, the difference lies in that BV is calculated from nitrogen absorbed whereas NPU is from nitrogen ingested. They can be used both in animals and humans but are experimentally difficult and are influenced by the previous nutritional state and the interaction of tested protein with other

foods. Peanut flour has a BV 56, which is lower than that of animal proteins, rice and corn proteins but higher than that of wheat gluten (Robinson *et al*., 1986). Khalil *et al*. (1983b) observed that NPU of wheat breads was significantly increased due to supplementation with peanut flour. BV and NPU of raw cowpeas range from 39.5-48.9 and from 34.9-40.7, respectively. Autoclaved cowpeas have increased values 48.4-58.2 and 44.9-52.9 for BV and NPU, respectively (Umoren *et al*., 1997). Replacing 310g/kg sorghum with cowpea results in an increase in BV and NPU values of the protein from 0.74 to 0.87 and from 0.58 to 0.69, respectively (Oyeleke *et al*., 1985).

The protein digestibility-corrected amino acid score (PDCAAS) has been adopted by FAO/WHO as the preferred method for the measurement of the protein value in human nutrition. PDCAAS determines protein quality by expressing the content of the first limiting essential amino acid of the test protein as a percentage of the requirement of the same amino acid for 2-5 year old children and then correcting it for the true fecal digestibility of the test protein. If the PDCAAS is greater than or equal to 1.00, the protein is a good source of essential amino-acids. Limitations of this method still exist relating to overestimation in the elderly, influence of ileal digestibility, urine excretion of nitrogen as ammonia, and antinutritional factors (Sarwar, 1997; Hoffman and Falvo, 2004). Peanut meal has a PDCAAS 0.52, which is lower than that of animal proteins, soybean protein, peas and lentils but higher than that of whole wheat and wheat gluten (U.S. Dairy Export Council, 2005). PDCAAS values for cowpea-based extrusion cooked maize/peanut weaning mixtures range from 0.72-0.82 with lysine being the first limiting amino acid, whereas when the amino acid profile of breast milk is used as reference for 0-1 year old infants, the PDCAAS values are significantly reduced and range from 0.46-

0.51 with the composite sulfur amino acid component (methionine+cystine) as the first limiting amino acid in the blend (Mensa-Wilmot *et al*., 2001). When the pig is used as a model for humans, decorticated, extruded cowpea has a PDCAAS of 0.87, surprisingly with leucine rather than methionie+cystine as the first limiting amino acid. It also has been suggested that correcting amino acid profiles by individual amino acid availability is a more appropriate approach to estimating protein quality since the extent of release and absorption of individual amino acids differs among each other and is different from that of protein. However, for the decorticated, extruded cowpea, only minor differences exist between the values provided by PDCAAS and amino acid availability-corrected amino acid scores (AAACAAS), and the limiting score is almost the same (Tuan *et al*., 1999).

Additional values of peanut and cowpea proteins and hydrolysates

Defatted peanut flour is a good carrier of L. *planetarium* P9 strain. The fermented product significantly increases the number of lactobacilli and decreases the number of enterobacteria in the fecal samples of mice, and thus can act as a probiotic food (Wang *et al*., 2007). New research shows fat-free peanut flour significantly lowers total cholesterol and low-density lipoprotein, the "bad" cholesterol in hamsters, while the high-density lipoproteins, the "good" cholesterol, remains steady (Gutierrez, 2008). Protein products not only provide bioavailable minerals, but also influence mineral metabolism in human. The latter has been attributed to the specific peptides and amino acids within the protein that form complexes with minerals to enhance transport and absorption. Peanut protein-fed rats have been found to have the higher apparent absorption rate of Cu, Fe and the higher apparent retention rate of Cu than the casein-fed rats and thus peanut protein may

make up for Cu and Fe deficiency (Chen *et al*., 2006). Proteins partially broken by heat, cooking, and enzymatic hydrolysis may increase mineral absorption and retention to a greater extent than intact proteins (Wapnir, 1998; Lönnerdal *et al*., 1999). A biologically important tripeptide, glutathione, contributes significantly to the absorption and transport of Cu and Fe (Wapnir, 1998; Etcheverry *et al*., 2006). Affinity purified copper-chelating peptides show higher contents of histidine and arginine in relation to the parent protein hydrolysates of sunflower obtained with pepsin and pancreatin (Megías *et al*., 2008). Histidine has also been proposed to enhance non-heme iron absorption (Swain *et al*., 2002; Etcheverry *et al*., 2006). Although we know peanut protein is rich in histidine and arginine, further research is necessary to identify the real enhancing factors of mineral absorption in peanut, or cowpea proteins, and to explain the mechanism. The metal-binding property of peptides derived from whole protein may also contribute to the *in vivo* antioxidant activity (Shahidi and Zhong, 2008).

Peanut and cowpea proteins could be made more valuable via enzymatic hydrolysis to create low molecule weight peptides. As compared to the peanut protein, Alcalase hydrolysate of peanut protein shows stronger inhibition of linoleic acid autoxidation, liver lipid autoxidation and H_2O_2 and Fe_2^+ induced oxidations *in vitro*. It is also an excellent 1,1-diphenyl-2-picrylhydrazyl free radical scavenger and displays strong reducing power *in vitro* (Chen *et al*., 2007). Unhydrolyzed peanut and cowpea proteins show little ACE inhibitory activity. Proteolytic peanut digests (Quist *et al*., 2009) and Alcalase hydrolysate of cowpea protein have been proved to effectively inhibit the *in vitro* ACE activity. Further research is required to purify the bioactive compounds from peanut and cowpea protein hydrolysates and then investigate their effects *in vivo*.

Occurrence of Angiotensin I converting enzyme (ACE)

Angiotensin I converting enzyme (ACE, EC 3.4.15.1) is a monomeric glycoprotein that is distributed in many tissues and biological fluids. There are two isoforms of ACE in human: somatic ACE (sACE) and germinal ACE (gACE). Somatic ACE is found in many types of endothelial cells and epithelial cells (Riordan, 2003). Germinal ACE or testicular ACE is present exclusively in germinal cells in the male testis. Although ACE is a type I integral membrane protein, it can also be released into extracellular fluids such as plasma, and seminal and cerebrospinal fluids as a soluble enzyme following a post-translational proteolytic cleavage by a membrane protein sheddase or secretase (Balyasnikova *et al*., 2002; Hooper and Turner, 2003; Parkin *et al*., 2004).

Gene Encoding and Structure of ACE

Somatic ACE and gACE are encoded by a single gene containing 26 exons. The promoter for sACE is situated in the 5'-flanking region of the first exon, whereas that for gACE is within intron 12, which results in different length for two isoforms. The longer sACE (150-180 kDa) is transcribed from exon 1 to exon 26, excluding exon 13, whereas the shorter gACE (90-110 kDa) is transcribed from exon 13 to exon 26. Exon 13 encodes a unique sequence for N-terminus of gACE, whereas downstream exons encode a common sequence for both isozymes (Hubert *et al*., 1990).

Somatic ACE and gACE both consist of a 28-residue hydrophilic C-terminal cytoplasmic domain, a 22-residue hydrophobic transmembrane domain that anchors the protein in the membrane and an N-terminal ectodomain (Figure 2.1) that is heavily

glycosylated with mannose, galactose, fructose, N-acetylneuraminic acid and N-acetyl-glucosamine (Murray and FitzGerald, 2007). The ectodomain of sACE is further divided into two similar domains (N-domain and C-domain) encoded by the homologous exons 4-11 and 17-24, respectively, and each domain contains an active His-Glu-X-X-His (HEXXH) sequence (Riordan, 2003). Somatic ACE is the only known metallopeptidase with two homologous active sites (Lew, 2004), which implies that there has been a gene-duplication event during evolution (Turner and Hooper, 2002). Except for a unique sequence constituting its N-terminus, gACE is identical to the C- terminal half of sACE

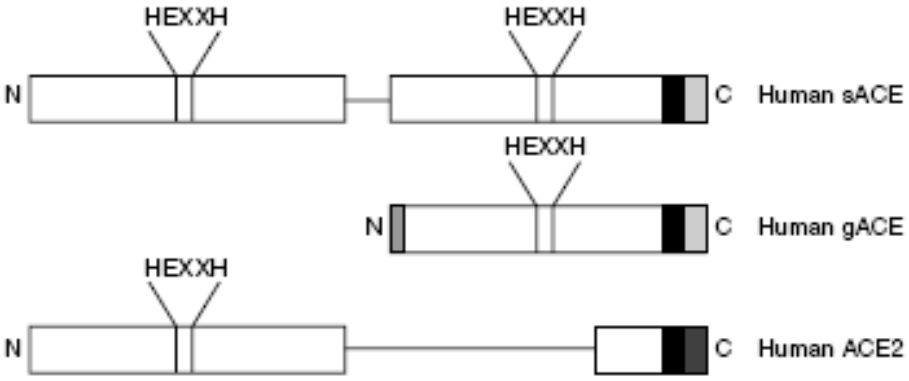

Figure 2.1 Schematic representation of the primary structure of several members of the ACE protein family (Riordan, 2003). Dimensions are not to scale. N, amino terminus; C, carboxyl terminus. Transmembrane domains are in black. The locations of the active-site zinc-binding motifs are indicated by HEXXH. The single lines are region of sequence with no similarity to other proteins. Except for its first 36 residues, the sequence of gACE is identical to that of the C-domain of sACE. Human gACE and sACE have the same carboxy-terminal transmembrane and cytosolic sequences, whereas ACE2 has a different transmembrane and cytosolic sequence.

(Natesh *et al*., 2003). Due to the cleavage of the membrane bound residues arginine and leucine by ACE secretase, soluble, circulating ACE thus lacks a transmembrane portion and a cytosolic domain (Balyasnikova *et al*., 2002).

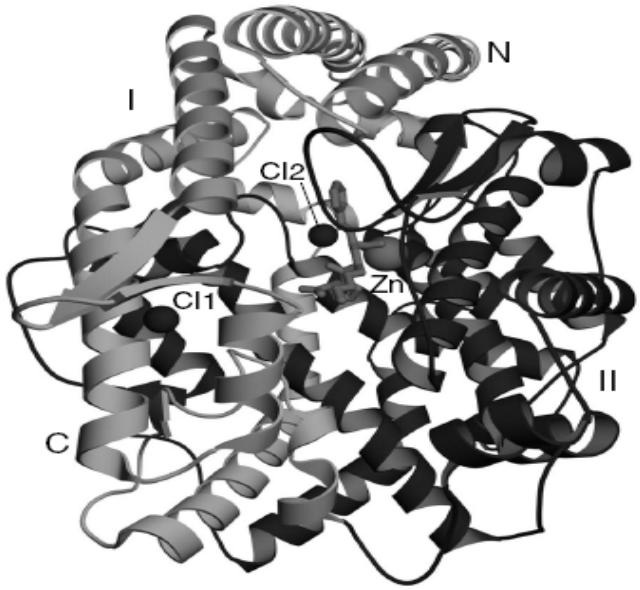

Figure 2.2 A schematic representation of the structure of deglycosylated, truncated human gACE in a complex with the inhibitor lisinopril (Riordan, 2003). The gACE molecule can be divided into two halves, subdomains I (light gray) and II (dark gray), that enclose the substrate-binding site. The active-site zinc atom is shown coordinated to lisinopril (in stick representation). Two bound chloride ions are designated Cl1 and Cl2. N, amino terminus; C, carboxyl terminus.

The three-dimensional structure by X-ray crystallography of a deglycosylated, truncated version of gACE (C domain of sACE) reveals a preponderance of α-helics with a zinc ion and two choride ions incorporated (Figure 2.2). A deep, narrow channel separates the molecule into two subdomains and the active site is located toward the

bottom of this channel. An N-terminal 'lid' is on the top of molecure which appears to allow only small peptide substrates access to the active site cleft. In fact, the structure bears little similarity to that of carboxypeptidase A (M14 family) on which the initial drug development of ACE inhibitors based. Instead, it resembles rat neurolysin (M3 family) and *Pyrococcus furiosus* carboxypeptidase (M32 family), despite sharing little sequence similarity with these two proteins (Natesh *et al*., 2003). Corradi *et al*. (2006) reported the crystal structure of N- domain of sACE. Similarly, it has an ellipsoid shape with a central groove dividing it into two sub-domains, one of which contains the N-terminal region that covers the central binding cavity. But the structure reveals difference in the active site and contains only one chloride ion, equivalent to chloride II of gACE. The 3D structures of C- (based on gACE) and N- domains provide an opportunity to design domain-selective ACE inhibitors that may exhibit new pharmacological profiles.

Catalytic Mechanism of ACE

According to the catalytic mechanism and the critical amino acid residue involved, peptidases are classified into four major types: serine, cysteine, aspartic and metallo-peptidases (Lew, 2004). ACE is a M2 family metallopeptidase (MA (E), “gluzincins”) (Rawlings and Barrett, 1993). Two histidines residues of the functional motif HEXXH and a third distant glutamate positioned 23-24 residues further towards the C-terminus are the ligands for the zinc cofactor (Coates, 2003). An activated water molecule complexed to Zn^{2+} serves as the nucleophile to attack the carbonyl group of the targeted peptide bond (Lew, 2004).

The activity of ACE is also chloride dependent. Chloride primarily activates the actives sites of ACE and enhances the binding of substrates (Riordan, 2003). Each active domain of ACE displays differences in the sensitivity to chloride activation (Wei *et al*., 1991). The activity of the C-domain of sACE depends highly on chloride ion concentration and is inactive in its absence, whereas the N-domain can be completely activated at relatively low concentrations of this anion and is still active in the absence of chloride (Jaspard *et al*., 1993; Araujo *et al*., 2000). Germinal ACE depends on chloride to a lesser extent compared to the C-domain of sACE (Riordan, 2003). Cushman and Cheung (1971) stated an optimal ACE activity of the rabbit rung acetone extract in the presence of 300 mM NaCl at pH 8.1-8.3.

The two active domains of sACE are also subtly different in the substrate specificity. They hydrolyze bradykinin almost equally but the C-domain active site can hydrolyze Angiotensin I, substrate P (Jaspard *et al*., 1993) and HHL (Corradi *et al*., 2007) more efficiently and the N-domain active site preferentially hydrolyzes Angiotensin (1-7) (Deddish *et al*., 1998), LH-RH (Jaspard *et al*., 1993), the hematoregulatory peptide N-acetyl-Ser-Asp-Lys-Pro (AcSDKP) (Rousseau *et al*., 1995), and Alzheimer amyloid β-peptide (Oba *et al*. 2005). Fuchs *et al*. (2008) proved that the C-terminal catalytic domain was the main site of Angiotensin I cleavage in mice. The differentiation of the catalytic specificity might be due to very subtle variation on substrate-specific amino acids (Araujo, 2000) and chloride-induced conformational alteration of active sites (Jaspard *et al*., 1993).

ACE acts as an exopeptidase to cleave dipeptides from the free C-termini of two typical subtrates angiotensin I and bradykinin. For certain substrates such as

cholecystokinin (Dubreuil *et al*., 1989), substrate P (Skidgel *et al*., 1984) and luteinizing hormone-releasing hormone (LH-RH) (Skidgel and Erdös, 1985), that have amidated C-termini, ACE not only displays exopeptidase activity, but also acts as an endopeptidase (Naqvi *et al*., 2005). The most prominent case of endopepidase activity is that ACE hydrolyzes the synthetic Alzheimer amyloid β-(1-40) peptide (Aβ-(1-40)) into four fragments with one as Aβ-(8-40) (Toropygin *et al*., 2008). Thus, ACE might have a more general impact on the metabolism of biologically active peptides than previously recognized (Turner and Hooper, 2002). The two most commonly used substrates Hippuryl-His-Leu (HHL) and N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG) for measuring ACE activity/inhibition *in vitro* only have the N-termini blocked and substrates with two termini blocked have been developed (Araujo *et al*., 2000).

Biological Impact of ACE

ACE was originally isolated in 1956 as a "hypertension-converting enzyme" (Skeggs *et al*., 1956). It plays an important role in the resin-angiotensin system (RAS) which regulates blood pressure and fluid homeostasis in human. The main effector molecule of the RAS, angiotensin II, is produced through an enzymatic cascade consisting of renin, an aspartic protease that first cleaves angiotensinogen to form the decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu, 1-10), and ACE that then further cleaves angiotensin I into the octapeptide angiotensin II (1-8) by removing the C-terminal dipeptide His-Leu (Lavoie and Sigmund, 2003). The resulting angiotensin II is a potent vasoconstrictor, stimulates the release of aldosterone and anti-diuretic hormone or vasopressin, and increases the retention of sodium and water and the

regeneration of rennin. These effects directly act in concert to raise blood pressure. A nonapeptide derivative of angiotensin I, des-Asp[1]-angiotensin I (2-10) which prevents infarction- and non-infarction-related cardiac injuries and disorders, can be cleaved the dipeptide His-Leu by ACE to produce Angiotensin III (2-8) (Murray and FitzGerald, 2007) which has 40% of the vasoconstriction activity of Angiotensin II. ACE also degrades angiotensin (1-9) to angiotensin (1-7) and further degrades this peptide to inactive angiotensin (1-5) (Figure 2.3). Angiotensin (1-7) actively opposes the vasoconstrictor and proliferative action of angiotensin II (Chen *et al*., 2005; Ferrario, 2006). In addition, ACE, also termed kininase II, inactivates the vasodilators bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, 1-9) and kallidin (Lys-bradykinin) in kallikrein-kinin system by cleaving the C-terminal dipeptide Phe-Arg. ACE eventually cleaves further its primary metabolite bradykinin (1-7) into the shorter fragment bradykinin (1-5) (Sivieri *et al*., 2007).

Through angiotensin II and aldosterone, ACE may also be implicated in the impairment of nitric oxide bioavailability and cell oxidative stress, augmenting the generation of reactive oxygen species and peroxynitrite (Jung *et al*., 2006; Imanishi *et al*., 2008). With the ability to hydrolyze neuropeptides such as enkephalin (Leung *et al*., 1992; Lund *et al.*, 1998), substrate P, neurotensin (Skidgel *et al*., 1984) and LH-RH, ACE may be involved in the functioning of brain and nervous system. ACE may affect the digestive system by hydrolyzing the peptide hormone CCK and gastrin (Dubreuil *et al*., 1989). The *in vivo* experiment conducted by Azizi *et al.* (1996) proved that ACE inhibition could increase the level of the natural stem cell regulator AcSDKP in plasma.

ACE may also affect susceptibility to Alzheimer's disease by degrading Aβ and preventing the accumulation of amyloid plaques *in vivo* (Hu *et al*., 2001).

Human ACE Homologue (ACE2)

ACE2, the first human homologue of ACE, is also a type I integral membrane glycoprotein expressed highly in heart, kidney and testis (Hooper and Turner, 2003; Elased *et al*., 2006). It appears to be susceptible to cleavage and secretion from the cell surface probably by a distinct secretase. It has 805 amino acids and shares about 42% sequence identity to the N- and C-domains of sACE (Turner and Hooper, 2002). ACE2 consists of a single active site domain that, by sequence comparison, more closely resembles the N-domain than the C-domain of sACE (Figure 2.1) (Hooper and Turner, 2003). In addition to the conserved zinc metallopeptidase consensus sequence HEXXH, there is a conserved glutamate residue 24 residues C-terminal to the second histidine of the zinc motif that serves as the third zinc ligand (Turner and Hooper, 2002). ACE2 is also activated by high concentration of chloride (Riordan, 2003).

Unlike sACE and tACE which are primarily dipeptidyl carboxypeptidases, ACE2 functions predominantly as a carboxypeptidase with a substrate preference for a C-terminal hydrophobic residue, cleaving a single residue leucine from angiotensin I, generating angiotensin (1-9) and a single residue phenylalanine from angiotensin II to generate angiotensin (1-7) (Figure 2.3). It can cleave des-Arg^{9}-bradykinin but fails to hydrolyze bradykinin which has a basic residue arginine at the C-terminus. ACE2 is like carboxypeptidase A (M14 family in MA clan) in action model but is different in active structure because the latter has a HXXE zinc-binding motif with the third ligand histidine

positioned 108-135 residues further towards the C-terminus. ACE2 is unaffected by inhibitors of either ACE (captopril, lisinopril and enalaprilat) or carboxypeptidase A (benzylsuccinate and potato carboxypeptidase inhibitor) (Turner and Hooper, 2002).

The major function of ACE2 is to counter-regulate ACE activity by reducing angiotensin II bioavailability and increasing angiotensin (1-7) formation (Hernάndez Prada *et al*., 2008; Sarkissian *et al*., 2008). The feedback regulatory mechanism of ACE2 (Figure 2.3), together with ACE determines the net concentration of two active components Angiotensin II and angiotensin (1-7) in RAS (Ferrario, 2006). Sarkissian *et*

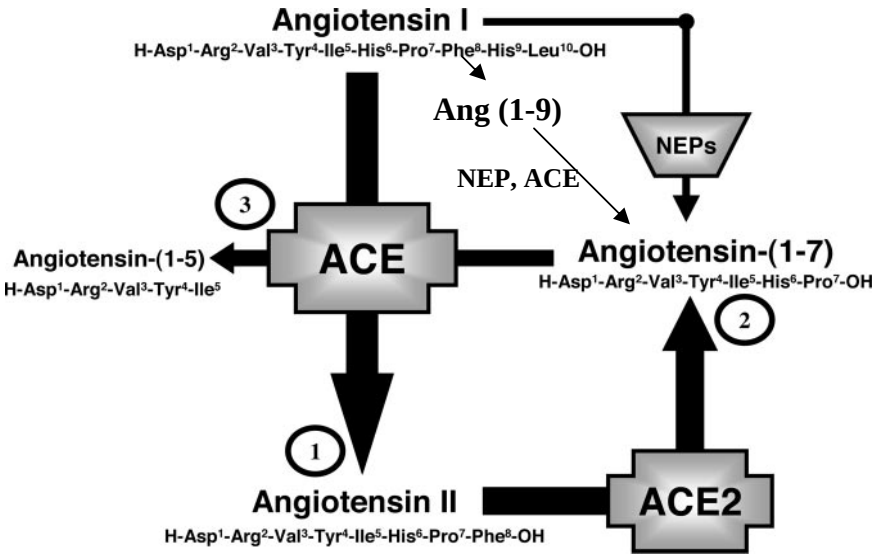

Figure 2.3 Schematic representation of feedback regulatory mechanism of ACE2 (Ferrario, 2006). Step 1: Ang II is formed through hydrolytic removal of His-Leu of the Ang I substrate by ACE; step 2: ACE2, acting as a monopeptidase, cleaves phenylalanine from Ang II to form Ang-(1-7); step 3: in turn, ACE then hydrolyzes Ang-(1-7) to form Ang-(1-5).

al. (2008) proved that the cardiac overexpression of ACE2 protects the heart from ischemia-induced pathophysiology. More functions of ACE2 were mentioned by Ferrario (2006). The discovery of ACE2 and its importance are encouraging efforts toward further understanding the complex and delicately balanced relationships among many bioactive peptides on which it may act (Riordan, 2003; Ferrario, 2006).

ACE inhibitors

Using the assumed mechanistic analogy to other zinc metallopeptidases, plus the knowledge that several snake-venom peptides potentiate the action of bradykinin by inhibiting ACE, efforts were undertaken to develop orally-active peptide analogs for potential use in the treatment of hypertension (Riordan, 2003). The first such compound, captopril or D-3-mercapto-2-methylpropanoyl-L-proline, is an analog of Ala-Pro sequence, with sulfhydryl as a strong chelating group of zinc ion. Its adverse effects, that were the same as caused by mercapto-containging penicillamine, prompted the design of non-sulfhydryl ACE inhibitors (Patchett *et al*., 1980). The results were two active inhibitors: enalaprilat and lisinopril. They both are essentially tripeptide analogues with a zinc-coordinating carboxyl group and a phenylalanine which occupies the S_1 groove in the enzyme. Lisinopril is a lysine-analogue of enalaprilat but it is hydrophilic with greater affinity than enalaprilat. The later compounds are all variations of the first three inhibitors, with most of the differences residing in the functionalities that bind the active-site zinc and the S_2' pocket (Figure 2.4). In addition to phosphonates, ketones have also been proven useful as chelators (Acharya *et al*., 2003).

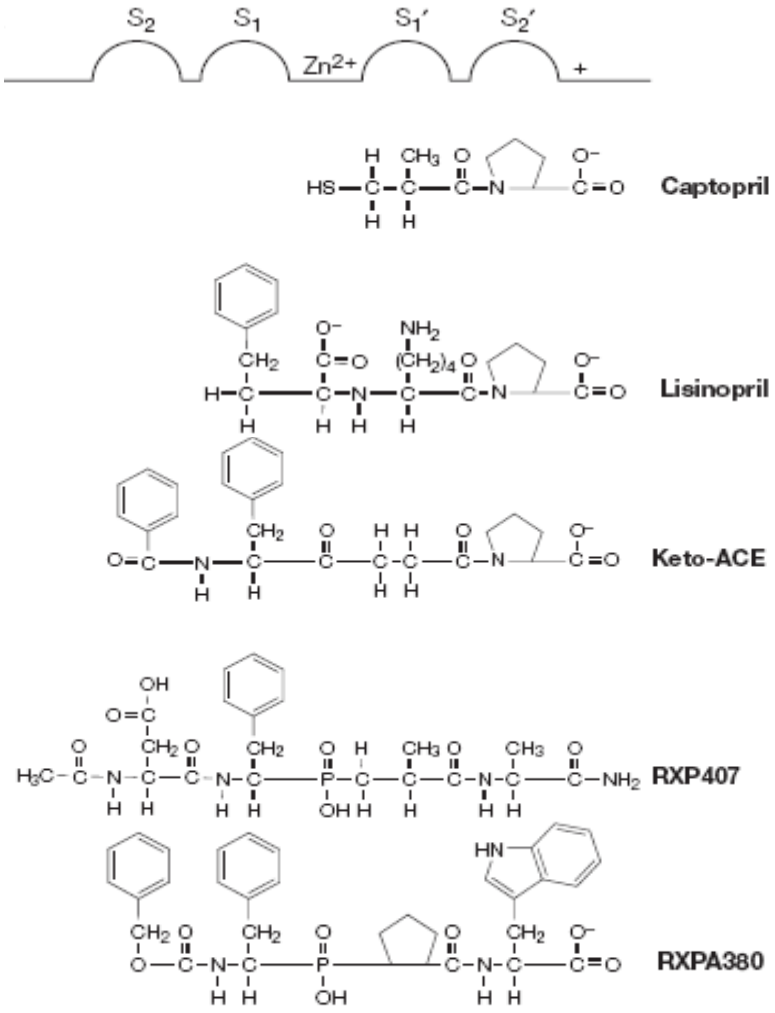

Figure 2.4 Classical models of inhibitors binding to the 'genetic' ACE active site (Acharya *et al*., 2003).

Currently, there are more than ten ACE inhibitors marketed in the United States that are widely used as the first-line therapy for cardiovascular diseases, including hypertension, heart failure, heart attack and left ventricular dysfunction (Lazar, 2005). According to the functional moiety, they are divided into three types: thiol (captopril), carboxylate (benazepril, enalapril, lisinopril, moexipril, perindopril, quinapril, ramipril, trandolapril) or phosphate (fosinopril). Some ACE inhibitors are now administrated

clinically as ethyl-ester prodrugs—which have good bioavailability, but are inactive in their own right. They are then converted to the active diacid molecules *in vivo* by esterases (e.g. benazepril to benazeprilat, eanlapril to enalaprilat, moexipril to moexiprilat, perindopril to perindoprilat, quinapril to quinaprilat, ramipril to ramiprilat, and trandolapril to trandolaprilat).

As a drug class, ACE inhibitors are very effective, have a relatively low incidence of side effects and are well-tolerated. A common side effect of ACE inhibitors is a dry cough appearing in 5-20% of patients and may result in the discontinuation of treatment. Another serious problem is angioedema which affects 0.1-0.5% of patients and can be life-threatening. The two side effects have generally been attributed to the altered concentrations of bradykinin (Acharya *et al*., 2003). Use of ACE inhibitors during the second and third trimesters of pregnancy is contraindicated because of their association with an increased risk of fetopathy, a group of conditions that includes oligohydramnios, intrauterine growth retardation, hypocalvaria, renal dysplasia, anuria, renal failure, and death. Exposure to ACE inhibitors during the first trimester of pregnancy may place the infant at increased risk for major congenital malformations (Cooper *et al*., 2006).

The initial drug development of clinical ACE inhibitors has been based on the assumption of an active site related to that of carboxypeptidase A but organized to remove a dipeptide rather than a single amino acid from the C-terminus of its substrate. It is now know that sACE has two active sites, neither of which resembles that of carboxypeptidase A, and that these sites are not identical. Clinical ACE inhibitors, however, show little discrimination between these two active sites (Riordan, 2003). Angiotensin I is hydrolyzed predominantly by C-domain of sACE *in vivo* (Junot *et al*.,

2001; Fuchs *et al*., 2004; Fuchs *et al*., 2008) but bradykinin is hydrolyzed by both active sites (Georgiadis et al., 2003), and therefore a C-domain selective inhibitor would allow some degradation of bradykinin by the N-domain and this degradation could be enough to prevent accumulation of excess bradykinin that has been observed during attacks of angioedma. That is, the C-domain selective inhibition could possibly result in specialized control of blood pressure with less vasodilator-related adverse effects (Acharya *et al*., 2003). Structure-activity study proved that the group substitution involving the phenyl ring and carbon chain at the sulfonyl and propionyl moieties of captopril are essential for better activity towards C-domain of ACE (San Juan and Cho, 2005). There is increasing evidence that N-domain of sACE is responsible for the *in vivo* degradation of the natural haemoregulatory hormone AcSDKP (Rousseau *et al*., 1995; Azizi *et al*., 1996; Junot *et al*., 2001). So N-domain selective inhibition might open up novel therapeutic areas. Two phosphinic tetrapeptides RXPA380 and RAP407 (Figure 2.4) have been found to be highly selective inhibitors of the C- and the N-domains of sACE, respectively (Acharya *et al*., 2003). The availability of the 3D structures of C- and N-domains of sACE may make the structure-based design of active site-specific inhibitors possible (Natesh *et al*., 2003; Corradi *et al*., 2006).

Plant food sources of ACE inhibitory peptides

ACE inhibitory peptides have been identified from various plant food sources including soybean, mung bean, sunflower, rice, corn, wheat, buckwheat, broccoli, mushroom, garlic, spinach, and wine (Table 2.1). Although the active peptides have not been sequenced, peanut (Quist *et al*., 2009), chickpea (Pedroche *et al*., 2002; Yust *et al*.,

2003), and potato (Pihlanto *et al*., 2008) protein hydrolysates do display strong ACE inhibitory activity. In silico gastrointestinal digestion of the highest scoring proteins, vicilin and albumin PA2 in pea directly releases a number of potent peptides, indicating that of pea protein which is a rich source of ACE inhibitory peptides (Vermeirssen *et al*., 2004a). A database survey shows that rye, barley and oats possess most of the known active peptides in their storage protein structure. Thus, cereal storage proteins can be potential sources of ACE inhibitory peptides (Loponen, 2004).

Production of plant food – derived ACE inhibitory peptides

ACE inhibitory peptides can be produced by solvent extraction, enzyme hydrolysis, and microbial fermentation of food proteins (Table 2. 1). Water soluble extracts from pulverized mushroom (Choi *et al*., 2001; Lee *et al*., 2004), and sonicated broccoli powder (Lee *et al*., 2006), contain higher ACE inhibitory activity than the organic solvent soluble extracts. Ma *et al*. (2006) used water at pH 9.0 to extract the defatted buckwheat flour and produced an active tripeptide, Gly-Pro-Pro. Water extract of garlic also provides several active peptides (Suetsuna, 1998). The most common way to produce ACE inhibitory peptides is through enzymatic hydrolysis of food proteins. The specificity of the proteolytic enzyme and process conditions influence the peptide composition of hydrolysates and thus their ACE inhibitory activities (van der Ven *et al*., 2002). The combination of pepsin-pancreatin or pepsin-chymotrypsin-trypsin is usually used to simulate the gastrointestinal degradation of food proteins in human. The pepsin treatment can not elicit the ACE inhibitory peptides effectively from buckwheat protein,

Table 2.1 Potent ACE inhibitory peptides derived from plant foods.

Source[a]	Parent protein	Enzyme	Amino acid sequence	IC_{50} (μM)	Reference
Soybean	Whole protein	Alcalase	DLP	4.8	Wu and Ding (2001)
			DG	12.3	
		Pepsin	YLAGNQ	14	Chen *et al*. (2003)
			FFL	37	
			VMDKPQG	39	
			IYLL	42	
		Fermentation	HHL[d]	2.2[b]	Shin *et al*. (2001)
			WL	29.9	Kuba *et al*. (2003)
			IFL	44.8	
	Protein isolate	Protease D3	NWGPLV	21	Kodera and Nio (2006)
			PNNKPFQ	33	
			YVVFK	44	
	Glycine	Protease P	VLIVP	1.69	Gouda *et al*. (2006)
Mung bean	Protein isolate	Alcalase	KDYRL	26.5	Li *et al*. (2006)
			KLPAGTLF	13.4	
Sunflower	Protein isolate	Pepsin-pancreatin	FVNPQAGS	6.9	Megías *et al*. (2004)
Rice	Protein isolate	Alcalase	TQVY[d]	18.2	Li *et al*. (2007)
Corn	Gluten	Alcalase	AY[d]	14.2	Yang *et al*. (2007)
Broccoli	Water extract	No enzyme	YPK	10.5[b]	Lee *et al*. (2006)
Mushroom	Water extract	No enzyme	GEP[d]	40[c]	Lee *et al*. (2004)
Garlic	Water extract	No enzyme	FY[d]	3.74	Suetsuna *et al*. (1998)
			NY[d]	32.6	
			NF[d]	46.3	

Table 2.1 (continued)

Source[a]	Parent protein	Enzyme	Amino acid sequence	IC_{50} (μM)	Reference
Wheat	Germ protein	Alcalase	TF	17.8	Matsui *et al*. (1999)
			LY	6.4	
			YL	16.4	
			AF	15.2	
			IY	2.1	
			VF	9.2	
			IVY[d]	0.48	
			VFPS	0.46	
			TAPY	13.6	
			TVPY	2	
			TVVPG	2.2	
			DIGYY	3.4	
			DYVGN	0.72	
			TYLGS	0.86	
			GGVIPN	0.74	
			APGAGVY	1.7	
	Gliadin	Pepsin-protease M	IAP[d]	2.7	Motoi *et al*. (2003)
Buckwheat	Whole protein	Pepsin-chymotrypsin	VK	13	Li *et al*. (2002)
		-trypsin	FY	25	
			YQY	4	
			PSY	16	
			LGI	29	
			ITF	49	
			INSQ	36	
	Water extract	No enzyme	GPP	6.25[b]	Ma *et al*. (2006)

Table 2.1 (continued)

Source[a]	Parent protein	Enzyme	Amino acid sequence	IC_{50} (μM)	Reference
Spinach	Rubisco	Pepsin-pancreatin	MRWRD[d]	2.1	Yang *et al*. (2003)
			MRW[d]	0.6	
			LRIPVA[d]	0.38	
			IAYKPAG[d]	4.2	
Wine	Wine concentration	No enzyme	IPPGVPY	17.5	Takayanagi and
			YYAPF	26.4	Yokotsuka (1999)
			WVPSVY	25.7	
			AWPF	18.3	

[a] the content in the blank position is the same as that in the last row of the same column; [b] IC_{50} values quoted are expressed as μg/ml; [c]IC_{50} values quoted are expressed as μg; [d] the *in vivo* assay has been conducted.

while pepsin treatment followed by chymotrypsin and trypsin results in a significant increase in the ACE inhibitory activity (Li *et al*., 2002). For pea protein, the highest ACE activity is reached early in the simulated stomach phase using pepsin treatment and the level is maintained during the simulated small intestine phase using trypsin-chymotrypsin treatment (Vermeirssen et al., 2004a). In other several studies, the plant protein hydyolysates generated during pepsin digestion had greater ACE inhibitory activities than those after subsequent digestion with pancreatin, which suggests that pepsin-produced inhibitory peptides are subsequently hydrolyzed during pancreatic hydrolysis (Yang *et al*., 2003; Megías *et al*., 2004; Yang *et al*., 2004; Lo *et al*., 2005). Commercially available bacterial and fungi proteases are also widely used in producing potent hydrolysates. Alcalase generates more potent ACE inhibitory hydrolysates than other studied proteases

from corn gluten (Yang *et al*., 2007), wheat germ (Matsui *et al*., 1999), potato tubers (Pihlanto *et al*., 2008), soy (Chiang *et al*., 2006), and peanut (Quist *et al*., 2009) proteins. The GC 106 (an acid protease from *Aspergillus niger* and commercialized by Genencor Co.) hydrolysates of wet- and dry-milled corn germ both reveal stronger ACE inhibitory activity than those from trypsin and thermolysin, while Flavourzyme treatment can not enhance the activity of either corn germ protein (Parris *et al*., 2008). In addition to the adequate match of the enzyme and protein sources, for a complete optimization of the hydrolysis process the influence of other parameters, like pH, temperature, enzyme to substrate ratio, hydrolysis time and their interactive effects on ACE inhibitory activity should also be considered (van der Ven *et al*., 2002). Research has been conducted to immobilize proteolytic enzymes. Compared to the soluble enzyme, Flavourzyme immobilized on highly activated glyoxyl-agarose support shows more thermal stability and produces less free amino acids in chickpea hydrolysis (Yust *et al*., 2007). Batch-type operations represent the most common mode for enzymatic hydrolysis of food proteins. However, there have been recent developments of protein digestion in a membrane reactor, in which hydrolysis of isolated soy protein is combined with partial purification of ACE inhibitory peptides from the reaction mixture through the use of membranes with varying molecular weight cut-offs. A continuous membrane reactor can result in higher productivities and more uniform products (Chiang *et al*., 2006).

ACE inhibitory activity has been found in traditional fermented soybean products, such as natto (Okamoto *et al*., 1995), tempeh (Gibbs *et al*., 2004), and Douchi (Zhang *et al*., 2006). Active peptides have been purified and sequenced from the fermented tofu Tofuyo (Kuba et al, 2003), soy paste (Shin *et al*., 2001), and soy sauce (Zhu *et al*., 2008).

However, fermentation can not fully hydrolyze soybean proteins to oligopeptides. Phosphoproteins, glycoproteins, and other post-translationally modified species that have a higher number of disulfide bridges are more difficult to cleave. The proteases in *Rhizopus* and *Bacillus* strains can only partly hydrolyze soybean protein (Gibbs *et al*., 2004). Further enzymatic degradations are needed to produce peptides with high activities (Gibbs *et al*., 2004; Zhang *et al*, 2006). The same is true of pea proteins where ACE inhibition of *Lactobacillus helverticus* and *Saccharomyces cervisiae* fermented pea protein was increased by subsequent pepsin/trypsin-chymotrypsin digestion (Vermeirssen *et al*., 2003)

Purification and sequence of plant food – derived ACE inhibitory peptides

ACE inhibitory peptides can be separated from a hydrolysate mixture by various kinds of membrane-based separation and chromatography techniques. Prior to the separation process, a peptide mixture is often subjected to precipitation, salting out, and solvent extraction (Shahidi and Zhong, 2008). Enzymatic hydrolysate of fermented soybean products can be treated using a mixture of water, acetonitrile and trifluoroacetic acid to extract active peptides (Gibbs *et al*., 2004). After centrifugation, the supernatant is filtered, usually by microfiltration under vacuum conditions, in order to remove enzymes and other insoluble components in the source material. The supernatant can also be applied to a column containing cation exchange resin which is washed with deionized water to remove impurities, and the desired peptides are eluted with ammonia solution (Suetsuna, 1998; Chen *et al*., 2003). Resin can be adopted to fractionate protein hydrolysates and peptides extracts before (Kuba, *et al*., 2005) or after (Wu and Ding,

2002) membrane separation. Kuba *et al* (2003, 2005) subjected soybean protein hydrolysates or peptide extracts from fermented tofu to different resins and then eluted with a step-wise gradient of ethanol to get strong ACE inhibitory fractions. Single- or multi-membrane separations have been used to isolate ACE inhibitory peptides. It is not always true that permeates from membranes with smaller molecular weight cut-offs (MWCO) have stronger ACE inhibitory potency. Compared to permeates from studied smaller MWCO membranes, permeates of potato liquid fraction (Pihlanto *et al*., 2008), soy protein hydrolysates (Wu and Ding, 2002; Chiang *et al*., 2006) from 10 kDa membrane had no significant difference in ACE inhibitory activities and therefore were selected for further purification.

Based on different properties of peptides, different chromatography techniques have been adopted. Among them, Reversed-phase HPLC is the most commonly used separation method. Frequently, reversed phase columns are packed with a chemically bonded octadecylsilyl coated silica; such columns are referred to as C-18 and are very non-polar. Other popular bonded columns have dodecylsilyl, octasilyl, or phenylsilyl packings. Gradient elution is usually practiced with gradually increased organic solvent (acetonitrile, methanol, propanol) concentration. The result is that the more polar components of peptide mixture elute first. Trifouroacetic acid (TFA), is often added to the eluting solvents to improve the chromatographic peak shape. Changing the concentration of TFA modifier can affect the resolution of peaks. RP-HPLC is usually coupled with a quantitative/qualitative analyzing instrument such as a UV detector or mass spectrometer. Among UV detectors, the photodiode array detector (PDA) is often used (Quist *et al*., 2009; Gibbs *et al*., 2004) and shows obvious advantages when

compared to the conventional UV detectors that are single channel detectors. PDA is a multichannel detector and can measure a spectrum of wavelengths simultaneously. It also gives more reproducible results than a conventional UV detector because the latter is operated by a stepper motor for selecting a specific wavelength and the moving part can affect the reproductibility (Choi, 2008). Recording a spectrum rather than a single absorbance provides, among other data, abundant comparisons within a symmetrical peak and may reveal the presence of two or more closely eluting components.

Other chromatography techniques include ion-exchange chromatography (IEC), capillary electrophoresis (CE), capillary isoelectric focusing (CIEF), and size-exclusion chromatography (SEC). IEC, CE and CIEF separate peptides based on their charge properties, while SEC is a separation method based on molecular size. SEC is also named gel filtration chromatography when operated in an aqueous mobile phase or gel permeation chromatography when performed in organic mobile phases (Wang and Gonzalez de Mejia, 2005; Shahid and Zhong, 2008). Li *et al.* (2002) obtained the di- and tri-peptide fraction having an average peptide length of 2.31 from the buckwheat digest though gel-permeation chromatography with a Superdex Peptide HR 10/30 column. More recently, affinity purification has been developed to separate ACE inhibitory peptides from alcalase hydrolysate of sunflower protein using immobilized ACE on an activated glyoxyl-agarose support (Megías *et al*., 2006b).

For unknown peptides, mass spectrometry methods are adopted to determine molecular mass and amino acid sequence. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are two main techniques for measuring molecular mass. For the former, the sample solution is pumped through a narrow,

stainless steel capillary; a high voltage is applied to the tip of the capillary which is located within the ionization source of the mass spectrometer, and a stream of nebulizing gas is introduced. As a consequence of the strong electric field, the sample emerging from the tip is sprayed into highly charged droplets. These droplets are then evaporated at the interface by the drying gas. Charged sample ions, free from solvent, are finally released from the droplets and detected by the analyzer. For the latter, the dried sample is introduced into the mass spectrometer where a laser is fired to desorb and ionize the sample. The matrix is used to absorb the energy needed for sample desorption and ionization from the laser and the analyzer separates ions according to their mass-to-charge ratio. A tandem mass spectrometer has more than one analyzer and generates structural information for a compound by fragmenting specific peptide ions and identifying the resulting fragment ions. This information can be then used to produce sequence information about the intact peptide (Ashcroft, 2009). For example, Ma *el al*. (2006) used ESI mode to get the molecular mass of ACE inhibitory peptides from mushroom. A HPLC coupled online to ESI mass spectrometry system was shown to be effective to sequence peptides with ACE inhibition activity purified from hydrolyzed corn gluten meal (Yang *et al*. 2007). The soy protein database (NCBI and SWISS-PROT) search combined with ESI mass/mass spectrometry (MS/MS) efficiently determined the amino acid sequence of peptides from soy protein hyrolysate (Kodera and Nio, 2006). MALDI is usually coupled to a time-of-flight (TOF) mass spectrometer because of its pulsed nature (Léonil *et al*., 2000). A MALDI-TOF/TOF tandem mass spectrometry can effectively provide mass spectra and tandem mass spectra for analyzing and sequencing

the purified ACE inhibitory peptides from mung bean protein hydroysate (Li *et al*., 2006).

Structural characteristics of plant food – derived ACE inhibitory peptides

Table 2.1 presents the source, hydrolyzing enzyme, sequence and other information about a large number of plant food-derived ACE inhibitory peptides. ACE inhibitory peptides are generally short sequences, which is in agreement with the results of Natesh et al. (2003) who showed that the active site of ACE cannot accommodate large peptide molecules. The C-terminal tripeptide strongly influences the binding of substrate or inhibitor to ACE. ACE appears to have a preference to a substrate or a competitive inhibitor containing hydrophobic (aromatic or branched side-chains) amino acids in the C-terminal tripeptide. ACE inhibition studies with dipeptides show that C-terminal phenylalanine, proline, tryptophan or tyrosine residues are the most effective in enhancing substrate binding (FitzGerald and Meisel, 2000). C-terminal lysine, leucine, isoleucine, valine may also contribute significantly to increasing ACE inhibitory activity of peptides (Murray and FitzGerald, 2007). It is suggested that arginine and phenylalanine residues in RSFCA are essential for a specific interaction with ACE and ACE inhibition (Kumada *et al*., 2007). In Table 2.1, several peptides have arginine in the penultimate position, which is in agreement with the suggestion of Rohrbach et al. (1981) that a positively charged amino acid in the penultimate position has a positive influence for peptide-enzyme binding. Structure-activity data suggest that a C-terminal arginine or lysine, with a positive charge on the guanidine or ε-amino group, respectively, seems to contribute substantially to ACE inhibitory potency. A possible interaction, thus, may

exist between the inhibitor and an anionic binding site of ACE that is distinct from the catalytic site. The removal of the arginine residue at the C-terminus can lead to essentially inactive peptide analogues (FitzGerald and Meisel, 2000; Murray and FitzGerald, 2007). ACE appears to require the L-configuration of amino acids at position three from the C-terminal (Murray and FitzGerald, 2007). That peptide conformation, i.e. the structure adopted in a specific environment, is also expected to contribute to ACE inhibitory potency. Due to substrate specificity difference between the two active sites of ACE, hydrophobic peptides have the superior binding to the N-terminal catalytic site, while hydrophilic peptides can only bind to the C-terminal catalytic site (Vermeirssen *et al*, 2004b; Shahidi and Zhong, 2008).

In vitro activity of plant food – derived ACE inhibitory peptides

Various methods have been used to quantify activity of plant food-derived ACE inhibitory peptides. In all cases, the enzyme is presented with peptide substrate, the hydrolysis of which is measured by detecting the formation of products. The extent to which inhibitors interfere with this reaction is a measure of their inhibitory power. The original method developed by Cushman and Cheung (1971) with minor modifications is the most widely adopted by later authors. The major development for this method lies in the selected product and the method of quantifying the product. The release of two products hippuric acid (HA) and L-His-L-Leu (HL) from the substrate hippuryl-L-histydyl-L-Leucine (Hip-His-Leu, HHL) hydrolysis by ACE is directly related to ACE activity. Instead of measuring the absorbance of extracted HA at 228 nm as in the original method, an aliquot of product mixture may be directly injected to HPLC system

to quantify the release of HA (Wu and Ding, 2002). Alternatively, the released HA is determined spectrophotometrically based on the specific colorimetric reactions of HA with 2, 4, 6-trichloro-s-triazine (TT) in dioxane (Megías, *et al*., 2004) or with benzene sulfonyl chlorine in the presence of quinoline (Li *et al*., 2006). In other modifications, the released HL is quantified spectrophotometrically based on the reaction of HL with 2, 4, 6-trinitrobenzene sulfonate (TNBS) (Matsui *et al*., 1999) or spectrofluorometrically by the fluorescent adduct between o-phthaldialdehyde (OPA) and HL (Motoi and Kodama, 2003). The inhibition mode of ACE-catalyzed hydrolysis of HHL is determined by Lineweaver-Burk plots. Competitive ACE inhibitory peptides are most frequently reported and have been identified from mushroom extracts (Choi *et al*., 2001; Lee *et al*., 2004), chickpea (Pedroche *et al*., 2002) and soy (Wu and Ding, 2002) protein hydrolysates. These inhibitors can bind to the active site to block it, or to the inhibitor binding site that is remote from the active site to alter the enzyme conformation such that the substrate no longer binds to the active site. Uncompetitive mechanism is also observed in chickpea peptides (Pedroche *et al*., 2002). Noncompetitive ACE inhibitory peptides have been isolated from sunflower protein hydrolysate (Megías et al., 2006a) and fermented soybean food (Kuba *et al*., 2003). The spectrophotometic assay using another synthetic tripeptide substrate N-[3-(2-furyl) acryloyl]-L-phenylalanylglycylglycine (FAPGG) blocked at the amino-terminus is gaining the popularity because it is simple and well suited to automation. The substrate has been used in quantifying ACE inhibitory activity of pea (Vermeirssen *et al*., 2002) and peanut (Quist *et al*., 2009) protein hydrolysates. In addition to the common assay of using soluble ACE, ACE immobilized on glyoxly-agrose has been developed to determine

ACE inhibition by sunflower peptide inhibitor. The immobilized ACE can be reused and has an increased thermal stability compared to the soluble enzyme (Megías *et al*., 2006a).

The potency of an ACE inhibitory peptide is usually expressed as an $IC_{50,}$ which is equivalent to the concentration of peptide inhibiting 50% of ACE activity (Murray and FitzGerald, 2007). An IC_{50} value is determined by regression analysis of ACE inhibition (%) versus peptide concentration (Megías *et al*., 2004) or ACE inhibition (%) versus log (peptide concentration) (Li *et al*, 2006, 2007). It can also be determined by fitting inhibitor concentration-ACE activity data to a four parametric logistic model using the Marquardt-Levenberg algorithm (Vermeirssen *et al*., 2002; Quist *et al*., 2009). The use of a variety of methods for measuring ACE inhibition and thus calculating IC_{50} has made it difficult for the exact comparison of IC_{50} values. The substrates and units of ACE activity within the assay may influence the determination of IC_{50} (Murray and FitzGerald, 2007). It should be pointed out that the unit shift from μg/ml to μM for the final pure peptides further complicates the direct comparison. Parris *et al*. (2008) got negative ACE inhibition values for Flavourzyme hydrolysates of corn germs according to their equation, which might be due to the failure to consider background sample absorbance. The peptide WL from soy glycinin protein shows two quite different IC_{50} values of 29.9μM (Kuba *et al*., 2003) and 65 μM (Kuba *et al*., 2005); another peptide FVNPQAGS from sunflower protein has also reported to have two distinct IC_{50} values of 6.9 μM (Megías *et al*., 2004) and 30.56 μM (Megías *et al*., 2006b).

Table 2.2 Bioactivity of plant food-derived ACE inhibitory peptides.

Peptides	Administration	Dose (mg/kg rat weight)	Activity	Reference
HHL	intravenous	5	decrease SBP 32 mmHg at 30 min	Shin et al. (2001)
TQVY	oral	30	maximum decrease of about 40 mmHg in SBP at 6 h	Li et al. (2007)
AY	oral	50	maximum decease of 9.5 mmHg in SBP at 2 h	Yang et al. (2007)
GEP	oral	1	decrease SBP about 36 mmHg at 2 h	Lee et al. (2004)
FY, NY, NF	oral	200	maximum decrease in SBP at 4h, 3h, 1h, respectively	Suetsuna (1998)
IVY	intravenous	5	decrease arterial BP 19.2 mmHg at 8 min	Matsui et al. (2000)
IAP	intraperitoneal	50	decrease SBP significantly at 1.5 h and 3h	Motoi and Kodama (2003)
MRWRD	oral	30	maximum decrease of 13.5 mmHg in SBP at 4h	Yang et al. (2003)
MRW	oral	20	maximum decrease of 20 mmHg in SBP at 2h	Yang et al. (2003)
LRIPVA	oral	100	no antihypertensive effect	Yang et al. (2003)
IAYKPAG	oral	100	maximum decrease of 15 mmHg in SBP at 4h	Yang et al. (2003)

SBP: systolic blood pressure.

Bioactivity of plant food-derived ACE inhibitory peptides

The *in vivo* assay of ACE inhibitory activity is generally conducted by measuring the blood pressure response in spontaneously hypertensive rats (SHRs) following intravenous injection, intraperitoneal or oral administration of the synthesized peptides (Table 2.2). Direct administration of angiotensin I has also been used to assess ACE inhibitory activity of peptides. For example, 15 mg/kg of IAP from wheat gliadin can inhibit the hypertensive activity of 50 mg/kg of angiotensin I with intravenous injection in SHRs (Motoi and Kodama, 2003). The wide variation in blood pressure responses may be due to variations in sample type, the dosage and administration, the mode of delivery and the method for the measurement of blood pressure (Murray and FitzGerald, 2007).

For example, triple injections of total 5 mg/kg of HHL from soy paste with an interval of 20 min can result in a significantly larger decrease in systolic blood pressure (SBP) of SHRs than a single injection of the same total amount of HHL. The lowering efficacy of triple injections on SBP is comparable to that of the synthetic antihypertensive drug, Captopril®. Unlike injection, the efficacy of orally administrated HHL might be changed by the accompanying digestive modification (Shin *et al*., 2001). Oral administration can convert certain prodrug-type peptides to true ACE inhibitors. For example, the antihypertensive effect of orally administrated IAYKPAG from spinach in SHR is probably a result of the antihypertensive activity of IAYKP, IAY, and KP (Yang *et al*., 2003). Conversely, hydrolysis of in vitro inhibitory peptides by peptidases in the brush border may inactivate them (Vermeirssen *et al*., 2004).

Interestingly, there appears to be some difference between the observed blood pressure reduction and the in vitro IC_{50} value. An example is that the peptide LRIPVA shows no antihypertensive effect after oral administration in SHRs at a dose of 100 mg/kg despite its potentACE inhibitory activity in vitro (IC_{50}= 0.38 μM). This may be due to the conversion of LRIPVA into peptides with very low ACE inhibitory activities (Yang *et al*. 2003). The IC_{50} values of food-derived ACE inhibitory peptides are about 1000-fold higher than that of the synthetic Captopril®, but there is no significant difference observed in the antihypertensive effect. Captopril® at a dose of 10 mg/kg results in a reduction of blood pressure in SHR of about 50 mmHg, while oral administration of 200 mg/kg of dipeptide purified from garlic exerts an antihypertensive effect of about 30 mmHg. The durations of the effect are not substantially different (Suetsuna, 1998; Vermeirssen *et al*., 2004b). The profiles of SBP versus oral

administration time are similar for 1 mg/kg of the peptide GEP from mushroom and Captopril® in SHR (Lee *et al*., 2004). These findings indicate that plant food-derived ACE inhibitory peptides have higher in vivo activity than would be expected from their in vitro activity when compared with antihypertensive drug Captopril®. It has been suggested that food-derived peptides might act via different antihypertensive mechanisms, possess higher affinities for tissues and are more slowly eliminated than the synthetic Captopril® (Vermeirssen *et al*., 2004b).

The transport of bioactive peptides affects their intestinal absorption and bioavailability. It has been proven that small peptides (di- and tripeptides) generated in diet can be absorbed across the brush border membrane by a specific peptide transport system and thus produce biological effects (Roberts *et al*., 1999). The caco-2 monolayer is generally used as a model to investigate intestinal transport. Two dipeptides, AF and IF, from salt-free soy sauce are transportable across the caco-2 cell monolayers and display ACE inhibitory activity. Kinetic studies show that IF possesses greater affinity toward the transport than AF (Zhu *et al*., 2008).

Further investigation into the *in vivo* and clinical antihypertensive effect of plant-food – derived ACE inhibitory peptides is necessary. Since it is based on a biological mechanism, evidence of in vitro ACE inhibitory activity is a good starting point (Vermeirssen *et al*., 2004b). ACE inhibitory peptides derived from plant foods that are consumed frequently can be used as components for functional foods (Vercruysse *et al*., 2005). As ACE possesses different functions in the human body, ACE inhibition may have additional implications than antihypertensive effects (Vermeirssen *et al*., 2004b).

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CHAPTER 3

APPLICATION OF A MICROPLATE KINETIC ASSAY TO THE DETERMINATION OF ANGIOTENSIN I CONVERTING ENZYME INHIBITORY ACTIVITIES OF ALCALASE HYDROLYSATES OF PEANUT AND COWPEA FLOURS[1]

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[1]Cuie Guang, Robert D. Phillips, to be submitted to the Journal of Food Chemistry.

ABSTRACT

When the amino-terminus protected tripeptide N-[3-(2-furyl)acryloyl]-L phenylalanylglycylglycine (FAPGG) was used as substrate to determine angiotensin I converting enzyme (ACE) activity/inhibition *in vitro*, different reference time intervals have been used to measure the absorbance change because of different reaction conditions and instruments. In this research, the kinetic reaction in which FAPGG is hydrolyzed by ACE to furylacryloylphenylalanine (FAP) and glycylglycine (GG) was closely examined through the use of an automatic microplate reader. The linearity for absorbance decrease was not maintained when the reaction time was extended beyond 15 min. The slopes of resulting kinetic curves for the first 15 min were used to calculate ACE inhibition. This was a rapid, simple and economic method to determine ACE activity/inhibition. For food protein hydrolysates, ACE inhibition began to plateau after peanut was hydrolyzed for 2.5 h and cowpea for 1 h.

Key words: Angiotensin I converting enzyme, FAPGG, Microplate reader, Protein Hydrolysates.

INTRODUCTION

Angiotensin I converting enzyme (ACE, EC 3.4.15.1) is a monomeric, membrane bound, zinc- and chloride dependent peptidyl dipeptidase. It catalyzes the conversion of the decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) to the octapeptide angiotensin II, by removing a carboxy-terminal dipeptide His-Leu (Riordan, 2003). The resulting angiotensin II is a potent vasoconstrictor, stimulates the release of aldosterone and anti-diuretic hormone or vasopressin, and increases the retention of sodium and water and the regeneration of rennin. In addition, ACE, also termed kininase II, inactivates the vasodilator bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) by cleaving the C-terminal dipeptide Phe-Arg. These effects directly act in concert to raise blood pressure and thus make ACE an ideal target in the treatment of hypertension.

Different substrates have been developed for spectrophotometric (Cushman and Cheung, 1971; Holmquist *et al*., 1979), fluorimetric or radiochemical assays (Friedland and Silverstein, 1977; Cheviron *et al.*, 2000; Sentandreu and Toldrá, 2006) to determine the *in vitro* ACE activity. The spectrophotometic assay using the synthetic tripeptide N-[3-(2-furyl) acryloyl]-L-phenylalanylglycylglycine (FAPGG) blocked at the amino-terminus is gaining the popularity because it is simple and well suited to automation. In general, the absorbance change (ΔA) at 340 nm between starting point and end point resulting from the hydrolysis of FAPGG to furylacryloylphenylalanine (FAP) and glycylglycine (GG) is measured to determine ACE activity. Different reference time intervals have been reported based on different reaction conditions and instruments. Vermeirssen *et al*. (2002) thought the reaction linearity was not maintained beyond the first 5 min; Murray *et al*. (2004) proved the initial rate of reaction was linear up to 30-min

incubation, after which the rate began to slow down; Erickson *et al*. (2003) measured the final absorbance after incubation for 1h. So each laboratory should establish ΔA for instruments and reassess it whenever the optics are changed (Buttery and Gee, 1992). An automatic microplate reader is able to conveniently provide a close examination of reaction kinetics. Herein we used a microplate kinetic assay to obtain the slope of reaction curves and therefore assessed the ACE inhibitory activities of Alcalase hydrolysates of peanut and cowpea flours.

MATERIALS AND METHODS

1. MATERIALS

ACE reagent N-[3-(2-furyl) acryloyl]-L-phenylalanylglycylglycine (FAPGG) was purchased from Trinity Biotech (Wicklow, Ireland). Angiotensin converting enzyme (ACE, EC 3.4.15.1) from rabbit lung and Alcalase 2.4 L (EC 3.4.21.62) were purchased from Sigma-Aldrich (St. Louis, MO).

Roasted peanut (Arachis hypogaea) flour purchased from Golden Peanut Company (Alpharetta, GA) was defatted by a series of extractions with hexane at room temperature, followed by grinding using a coffee bean grinder (Series CBG5, Black & Decker Co., Towson, MD). Raw cowpea (Vigna unguiculata) flour was roasted at 177°C for 10 min and then ground using the same grinder. Nitrogen contents were determined using a Leco FP2000 (Model 602-600, LECO Co., Warrendale, PA) and proteins were calculated to be 55.8% (N x 5.46) and 23.5% (N x 6.25) for peanut and cowpea flours, respectively. The resulting peanut and cowpea flours were used as the starting materials.

2. METHODOLOGY

Microplate kinetic assay of ACE activity

The ACE reagent contains 0.005 mmol FAPGG with stabilizer and buffer to ensure the reaction takes place at pH 8.1-8.3. It was reconstituted with 5 ml instead of indicated 10 ml deionized water in order to maintain the substrate concentration after addition of a volume of inhibitory compound (Vermeirssen *et al*., 2002). A 125 µl volume of the resulting substrate was added to designated microwells of a 96-well Costar microplate (Corning, NY), along with 125 µl of deionized water (control) or protein hydrolysate; the plate was covered by a lid, and pre-incubated at 37ºC in a FLUOstar Optima microplate reader (BMG LABTECH, Durham, NC) for 10 min. A 250 µl aliquot of deionized water was used as blank.

After incubation, the lid was removed and the operation was initiated using OPTIMA 2.10R3 (BMG LABTECH, Durham, NC) control software with the instrument settings as shown: positioning delay, 0.5s; measurement start time, 2.5 s; No. of flashes, 20; cycle time, 60 s; excitation filter, 340 nm; emission filter, empty; shaking mode, double orbital; shaking width, 2mm; additional shaking, 5 s before each cycle; injection volume, 25 µL; pump speed, 420 µL/s; injection cycle, 1; injection start time, 0.0 s; shaking after injection, 1 s; incubator temperature, 37°C. A 25 µl aliquot of ACE (0.005 units; 18.18 units/l reaction mixture) was injected automatically by the onboard injector with the help of the designated pump to each microwell to start the reactions. Absorbance was recorded every minute for 15 min.

The ACE inhibition (%) was calculated as follows:

$$\text{ACE inhibition (\%)} = 100\text{-}100\times S_{inhibitor}/S_{control}$$

where $S_{inhibitor}$ was the slope of kinetic curve in the presence of hydrolysate, $S_{control}$ was the slope without hydrolysate inhibitor.

Preparation of ACE inhibitory samples

Hydrolysis was performed at the following parameters: substrate concentration 1.25% for peanut flour and 2.5% for cowpea flour; enzyme/ substrate ratio 0.3 AU/g peanut flour and 0.2 AU/g cowpea flour; pH 7.5; temperature 60°C. The reacting mixture was pre-incubated in a water bath for 20 min and then Alcalase was added. During the reaction, pH was monitored and maintained by adding drops of 1N NaOH. Hydrolysis was terminated by boiling the hydrolysate for 15 min. The resulting hydrolysates were centrifuged at 27,000 *g* for 15 min at 10°C and then filtered through a 0.2 µm Millipore nylon filter (Bellerica, MA).

RESULTS AND DISCUSSION

Microplate kinetic assay of ACE activity

The slope of a reaction curve describing absorbance versus time is a direct measure of the enzyme activity (Shalaby *et al.*, 2006). Figure 3.1 shows the kinetic curve after seven replications for FAPGG hydrolysis to FAP and GG. After the microplate reader injected ACE into a microwell, it recorded the absorbance immediately for that microwell. Due to inhomogeneity, the absorbance at the beginning (0 min) was excluded before calculation of the slope. The absorbance decreases with reaction time observed, were in agreement with the result of Murray *et al*. (2004) who quantified the absorbance change at 340 nm of a range of solutions at an overall concentration of 1 mM containing

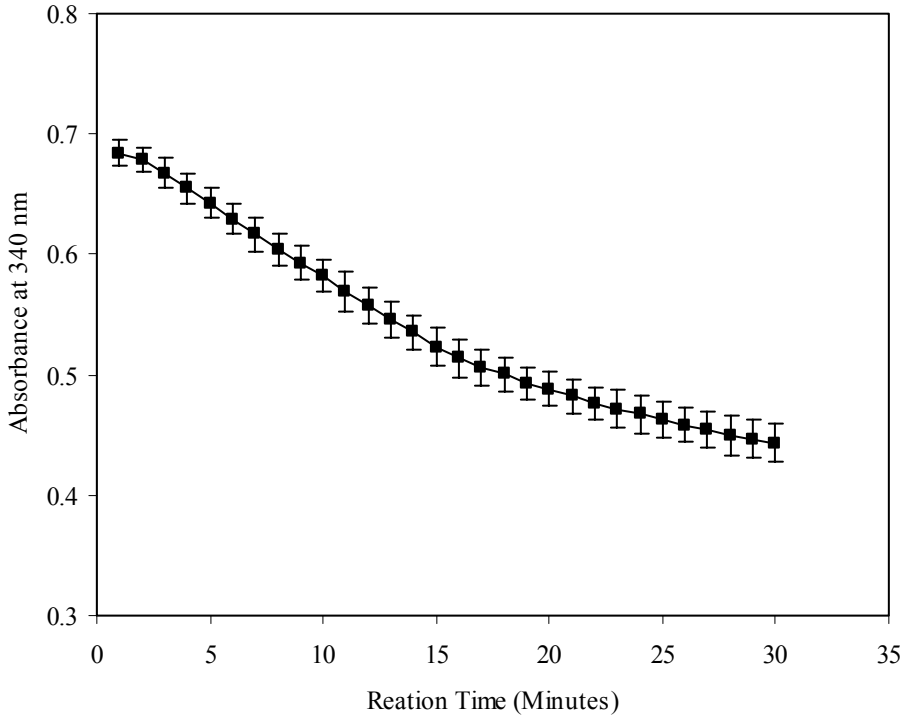


Figure 3.1 The kinetic curves of FAPGG hydrolysis by ACE to FAP and GG. Data represent the average ± standard deviation of seven experiments.

Table 3.1 The effect of reaction time on curve slopes and R^2.

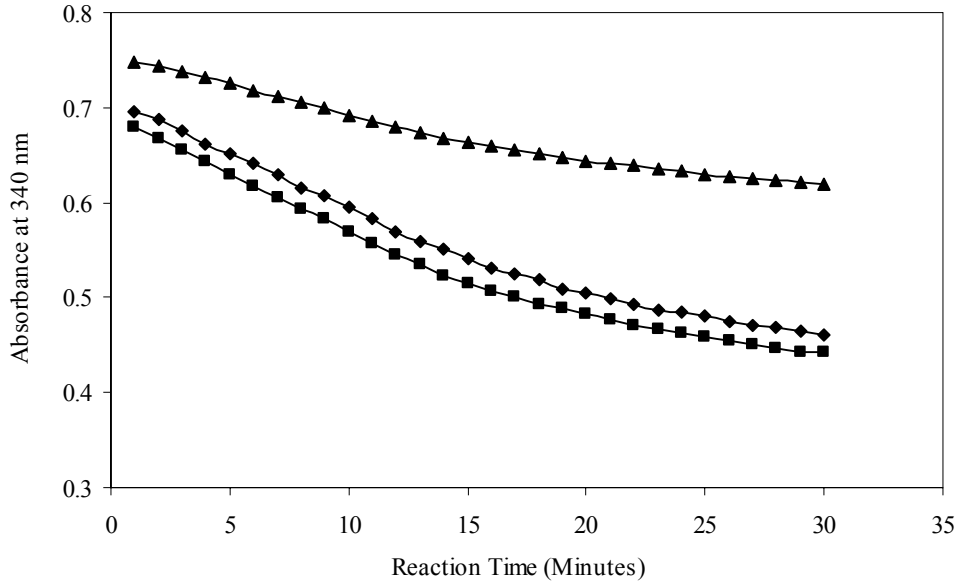
Time Interval (Minutes)	|Slope|	R^2
1-5	0.0107	0.9816
1-10	0.0119	0.9965
1-15	0.0119	0.9989
1-20	0.011	0.9933
1-25	0.0098	0.9785
1-30	0.0087	0.9612
16-20	0.0066	0.9971
16-30	0.005	0.988
26-30	0.0037	0.9923

decreasing concentrations of FAPGG (1.0-0.0 mM) and increasing concentrations of an equimolar mixture of FAP and GG (0.0-1.0 mM). Further investigation showed that the linearity wasn't always maintained. In Figure 3.1 when the reaction time was extended from 15 to 20, 25 or 30 min, absolute values of slopes for reaction curve was consequently reduced from 0.0119 to 0.011, 0.0098 or 0.0087 (Table 3.1). The R^2 value decreased from 0.9989 to 0.9933, 0.9785 or 0.9612. Absolute values of slopes for the interval 16-20 min and the interval 26-30 min were only 0.0066 and 0.0037, respectively. As the reaction goes to completion, the rate begins to slow down. Also, the resulting product GG can also exert inhibitory effect on ACE (Cheung *et al*., 1980). For the first 5 min, the slope 0.0107 and the R^2 value 0.9816 were lower than those for 10 min (slope= 0.0119, R^2 =0.9965) and 15 min. This may be due to mixture inhomogeneity, temperature difference between injected ACE and pre-incubated solution, or unsteadiness of instrument at the beginning of reaction. So the slope of reaction curve was preferably calculated using the first 15 min. The microplate kinetic assay clearly shows the reaction process and therefore the results are able to be closely examined.

ACE inhibitory activities of Alcalase hydrolysates of peanut and cowpea flours

When the inhibitors were present as shown in Figure 3.2, each hydrolysate produced background absorbance. Hyrolysates with hydrolysis time 0 h increased the absorbance almost equally at each recorded point. When the hydrolysis time was above 0 h, a decrease in absolute value of slope was observed. Figure 3.3 shows ACE inhibitory potency of Alcalase hydrolysates of peanut and cowpea flours having different hydrolysis

a



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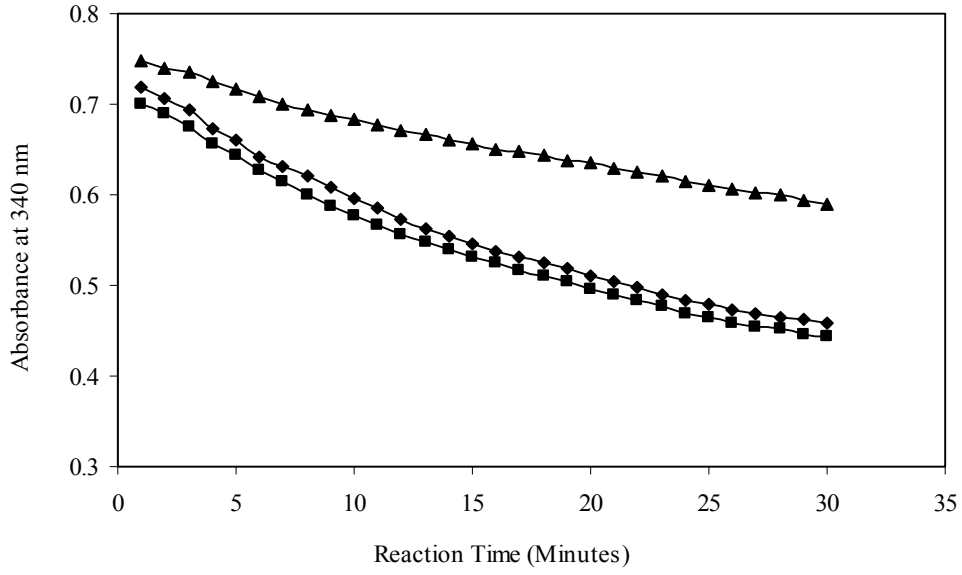
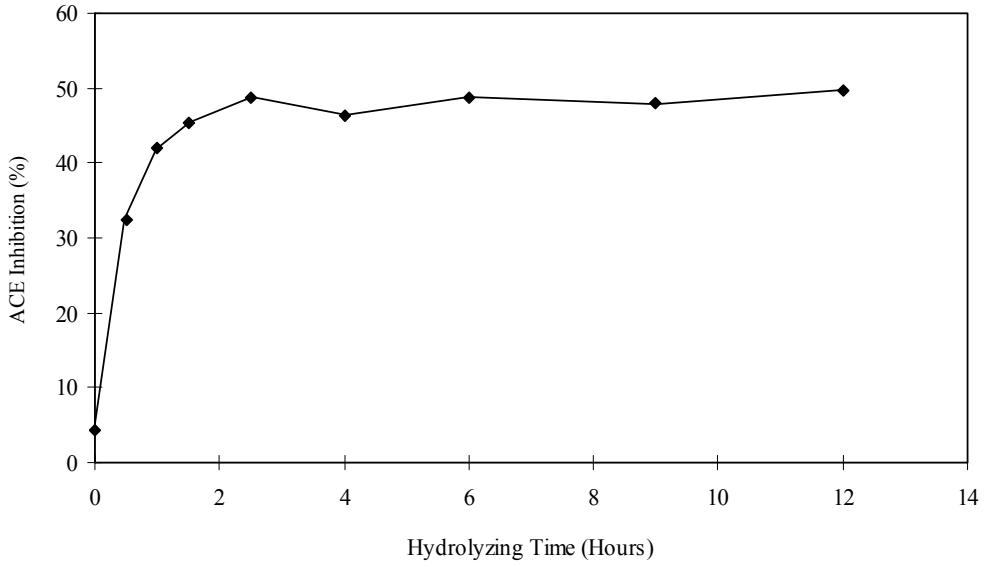


Figure 3.2 The kinetic curves of FAPGG hydrolysis by ACE to FAP and GG in the presence of Alcalase hydrolysates of peanut (a) and cowpea (b) flours: (■) no hydrolysates; (♦) 0 hour hydrolysates; (▲) 12 hour hydrolysates. The original hydrolysates were diluted to 1:30 (V/V) to act as inhibitors.

a



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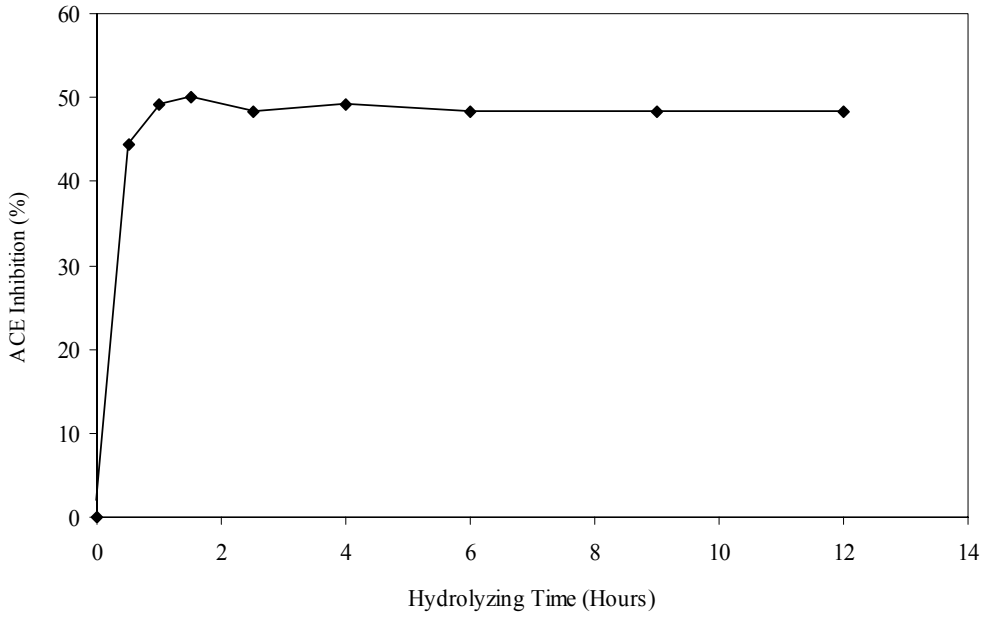

Figure 3.3 ACE inhibition by Alcalase hydrolysates of peanut (a) and cowpea (b) flours having different hydrolysis time. ACE inhibition was based on the hydrolysates diluted to 1:30 (v/v).

times. The trace ACE inhibition by peanut with hydrolysis time 0 h may be due to the failure to instantly inactivate Alcalase after its addition or the pyrolysis of peanut protein to yield fragments with low inhibitory activity. Alcalase could generate most of ACE inhibitory activity for the first 30-60 min, and ACE inhibition began to plateau after peanut was hydrolyzed for 2.5 h and cowpea for 1 h. Similar trends have also been observed in hydrolysis of soybean (Chiang *et al*., 2006) and potato (Pihlanto *et al*., 2008) proteins by Alcalase. When compared to the curve of degree of hydrolysis (DH) vs. hydrolysis time for peanut protein (Quist *et al*., 2009), ACE inhibition appeared to plateau earlier, which indicates that ACE inhibition is not completely related to DH (Chiang *et al*., 2006) and there may be a steady state between the generation and degradation of inhibitory peptides during this period. ACE inhibitory peptides from the prolonged hydrolysis by Alcalase, an enzyme with rather broad specificity, might be resistant to digestive enzyme and thus might be allowed for absorption in the GI tract (Pedroche *et al*., 2002).

CONCLUSION

In the present research we have established the assay for determining ACE inhibition using an automatic microplate reader. Compared to manual ACE assay using cuvets as presented by Vermeirssen *et al.* (2002), this assay dramatically decreases ACE reagent, ACE and sample volumes as well as analysis time. The slope in a kinetic assay is a more robust estimate of ACE activity/inhibition than a two-point mode in which only initial and final absorbances were used. Additionally, the method avoids the consideration of absorbances of hydrolysate samples and ACE themselves in the end

mode in which N-hyppuryl-His-Leu (HHL) was used as substrate (Li *et al*., 2005, Guo *et al*., 2008). According to Parris *et al*. (2008), a negative value appeared when HHL was used as substrate and the absorbance of the resulting product hyppuric acid (HA) was measured to calculate ACE inhibition (%) for Flavourzyme hydrolysates of wet- and dry-milled corn germ. This might be due to the failure to consider the effect of background sample on absorbance. Here peanut and cowpea proteins are proved to be good sources of ACE inhibitors.

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CHAPTER 4

EFFECTS OF HYDROLYSIS FACTORS, pH, TEMPERATURE AND ENZYME TO SUBSTRATE RATIO, ON ACE INHIBITORY ACTIVITES OF ALCALASE HYDROLYSATES OF PEANUT AND COWPEA FLOURS[1]

[1]Cuie Guang, Robert D. Phillips, to be submitted to the Journal of Food Chemistry.

ABSTRACT

Peanut and cowpea are two important plant food protein sources. The peptide compositions of their protein hydrolysates determine the ACE inhibitory potency and depend on the specificity of the proteolytic enzyme and the hydrolysis conditions adopted for producing hydrolysates. In this research, the effects of three hydrolysis factors, pH, temperature and enzyme to substrate (E/S) ratio, on ACE inhibitory activities of Alcalase hydrolysates of peanut and cowpea flours were investigated systematically using response surface methodology. The response surface models were established and the significance of each term in models was analyzed. Hydrolysis factors, temperature and E/S ratio, showed significant effects, while pH had no significant effect over the range studied. Their optimal values were determined and were found to result in no great difference in ACE inhibition when compared to the hydrolysis conditions at the chosen central point.

Key words: Angiotensin converting enzyme, Peanut, Cowpea, Protein hydrolysates, Alcalase, Response surface methodology.

INTRODUCTION

Peanut (*Arachis hypogeae*) and cowpea (*Vigna unguiculata*), two important grain legumes, are both rich sources of protein. Crude protein content of whole seed peanuts ranges between 22 and 30% (Pancholy *et al*., 1978) and in cowpea cultivars, protein content ranges from 20-34.2% with a mean of 24% (Ofuya and Akhidue, 2005). Many biological peptides with health benefits, which are inactive within the parent proteins, may be liberated by enzymatic digestion. Various food protein hydrolysates have been shown to be good sources of ACE inhibitory peptides (Vercruysse et al., 2005; Murray and FitzGerald, 2007). The specificity of the proteolytic enzyme and process conditions influence the peptide composition of hydrolysate (van der Ven et al., 2002) and thus the ACE inhibitory potency.

Alcalase (EC 3.4.21.62) is produced from the fermentation of *Bacillus licheniformis*. It is a member of the S8 family serine endopeptidase, and uses a catalytic triad containing Asp, His and Ser amino acid residues and a ping-pong catalytic mechanism to break down the targeted peptide bonds (Rawlings and Barrett, 1993) with a rather broad specificity. Compared to other studied proteases, Alcalase generates more potent ACE inhibitory hydrolysates from corn gluten (Yang *et al*., 2007), wheat germ (Matsui *et al*., 1999), potato tubers (Pihlanto et al., 2008), soy (Chiang *et al*., 2006), and peanut (Quist *et al*., 2009) proteins than other studied enzyme systems. In addition to the adequate match of the enzyme and protein sources, for a complete optimization of the hydrolysis process the influence of factors, like pH, hydrolysis temperature, enzyme to substrate ratio, and their interactive effects on ACE inhibitory activity should be also considered (van der Ven *et al*., 2002). Alcalase has an optimum hydrolysis temperature

range between 55°C and 70°C and a pH between 6.5 and 8.5 (Sigma-Aldrich, Inc., St. Louis, MO). A diversity of hydrolysis parameters have been adopted to hydrolyze different food proteins.

Response surface methodology has been proved to be a valuable tool for developing a mathematical model, describing the effect of the independent variables alone and in combinations, and simultaneously optimizing several process factors in hydrolysis (van der Ven *et al*., 2002; He *et al*., 2005; Guo *et al*., 2008). In this study, the effects of three hydrolysis factors, i.e. pH, temperature, and enzyme to substrate ratio, on ACE inhibitory activity of Alcalase hydrolysates of peanut and cowpea flours were investigated systematically using response surface methodology.

MATERIALS AND METHODS

The starting materials and microplate kinetic assay of ACE inhibition were the same as described in Chapter 3.

Enzymatic hydrolysis of peanut and cowpea flours

Experiments to investigate the effects of hydrolysis variables in the range given in Table 4.1 were conducted according to the experimental design depicted in Table 4.2. The reacting mixtures with substrate concentration 1.25% (w/v) for peanut flour and 2.5% (w/v) for cowpea flour were pre-incubated at the designated temperature in a water bath for 20 min. Alcalase was added and the reaction was allowed to proceed for 3 h under constant shaking. During reaction, pH was monitored and maintained by adding drops of 1N NaOH. Hydrolysis was terminated by boiling the hydrolysate for 15 min.

Table 4.1 Factors and levels of Box and Behnken design for peanut and cowpea flours hydrolysis.

Independent variables	Symbols		Levels		
	Uncoded	coded	-1	0	1
Hydrolysis pH	pH	x_1	6.5	7.5	8.5
Hydrolysis Temperature (°C)	T	x_2	45	60	75
Enzyme/substrate (AU/g flour)	E/S	x_3	0.01 (0.008)[a]	0.3 (0.2)[a]	0.59 (0.392)[a]

[a]parameters in parentheses are for cowpea flour.

The resulting hydrolysate was centrifuged at 27000×g for 15 min at 10°C and then filtered through a 0.2 μm Millipore nylon filter (Bedford, MA).

Experimental design and statistical analysis

Response surface methodology (RSM) was used to evaluate effects of hydrolysis variables pH, temperature and enzyme/substrate ratio (AU/ g flour) on ACE inhibitory activities of peanut and cowpea hydrolysates. A Box-Behnken statistical screening design with three factors and three levels is suitable for exploring quadratic response surface and constructing a second-order polynomial model that describes linear, interaction and quadratic effects of hydrolysis variables. Table 4.1 and Table 4.2 give the uncoded and coded levels and experimental design. The variables were coded according to the following equation:

$$x_i=(X_i-X_0)/\Delta x_i$$

where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point, and Δx_i is the step change value. ACE inhibition data were subjected to a second-order

Table 4.2 Box-Behnken design matrix and the response of ACE inhibition (%) for Alcalase hydrolysates of peanut and cowpea flours.

Independent variables			Response	
pH	T (°C)	E/S (AU/g flour)	ACE inhibition (%)[a]	
(x_1)	(x_2)	(x_3)	Peanut	Cowpea
-1	-1	0	40.3	43.8
1	-1	0	43.8	45.5
-1	1	0	45.8	46.6
1	1	0	43.1	47.1
0	0	0	54.2	55.2
-1	0	-1	47.1	44.3
1	0	-1	46.9	45.1
-1	0	1	50.1	53.5
1	0	1	51.4	52.3
0	0	0	54.8	54.3
0	-1	-1	35.2	38.2
0	1	-1	40.4	40.7
0	-1	1	46.6	47.4
0	1	1	41.9	45.8
0	0	0	55.1	54.9

[a] Original hydrolysates were diluted to 1:25 (v/v) for measurement of ACE inhibition.

multiple regression analysis using the least square regression methodology to predict the parameters for the mathematical equation below:

$$Y=\beta_0+\sum\beta_i x_i+\sum\beta_{ii}x_i^2+\sum\beta_{ij}x_i x_j$$

where Y is the response (ACE inhibition (%)), β_0 is the value of the fixed response at the central point of the experiment (intercept), β_i, β_{ii} and β_{ij} are the linear, quadratic and crossproduct regression coefficients, respectively. Statistical analysis was performed with SAS 9.1 (SAS Institute Inc., Cary, NC). Experiments were run in duplicate and analytical determinations in triplicate. The plots are shown as an averaged response.

RESULTS AND DISCUSSION

Response surface modeling of hydrolysis of peanut and cowpea flours by Alcalase

Hydrolysates were diluted to 1:25 (v/v) to measure the ACE inhibition, which made the inhibition data around 50%. After statistical analysis of variance, the second-order polynomial equations for peanut (Y_1) and cowpea (Y_2) are given below:

$$Y_1=54.7+0.24x_1+0.66x_2+2.55x_3-1.8x_1^2-1.55x_2x_1-9.65x_2^2+0.375x_3x_1-2.48x_3x_2-4.03x_3^2$$

$$Y_2=54.8+0.23x_1+0.66x_2+3.84x_3-1.64x_1^2-0.3x_2x_1-7.41x_2^2-0.5x_3x_1-1.03x_3x_2-4.36x_3^2$$

These coefficients are the parameter estimates based on coded data. The lack-of-fit tests, which measure the fitness of the models obtained, resulted no significance (p>0.05) (Table 4.3), indicating that the models are sufficiently accurate for predicting ACE inhibition for any combination of independent variable values within the ranges studied. The closer the determination coefficient R^2 value is to 1.00, the better the model can predict the response. R^2 values were calculated to be 0.987 and 0.991, and implied that 98.7% and 99.1% of the behavior variation can be explained by the fitted models over the range of factor values tested for peanut and cowpea, respectively. They are high and

satisfactory R-square values. The total models' p values ($p<0.05$) indicated that the models are significant.

Table 4.3 Variance analysis for the response of ACE inhibition (%)

	Pr >F								R^2
	Residual	Regression				Factor			
	lack of fit	Linear	Quadratic	Crossproduct	Total model	x_1	x_2	x_3	
Peanut	0.097	0.0066	<0.0001	0.0183	0.0004	0.0673	0.0001	0.0014	0.9868
Cowpea	0.1822	0.0002	<0.0001	0.1505	0.0001	0.0691	0.0001	0.0001	0.9914

Table 4.3 shows that the over-all linear, quadratic and cross products are all significantly effective at a 5% significant level except the crossproducts for cowpea. The effect of each term is depicted in the pareto charts (Figure 4.1). The length of each bar represents the standardized effect, which equals the magnitude of the t statistic that would be used to test the statistical significance of that effect and is calculated by dividing each coefficient estimate based on the raw data by its standard error. These bars are sorted from the most significant at the top to the least significant at the bottom. A vertical line is drawn at the location of the 0.05 critical value for Student's t. Any bars that extend to the right of that line indicate significant effects at the 5% level. The chart shows that the response surface parameters estimates are significant except for the interaction term of pH and E/S ratio of peanut hydrolysis and three cross terms of cowpea hydrolysis, indicating these interaction terms can be dropped from the models. Obviously, the linear and quadratic terms of hydrolysis temperature are the most effective on ACE inhibitory activities of peanut and cowpea hydrolysates, which is agreement with the results of van der Ven *et al*. (2002).

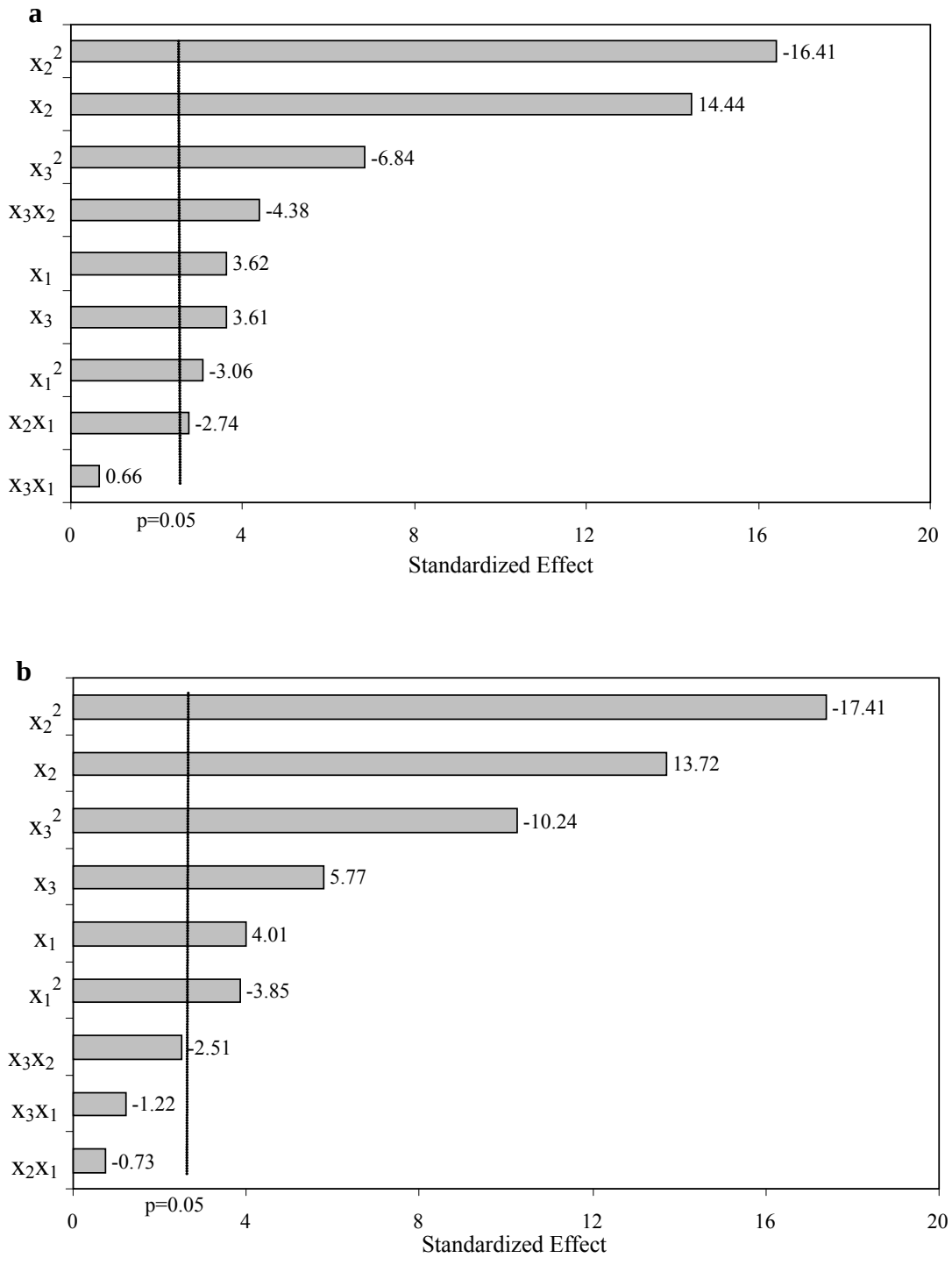
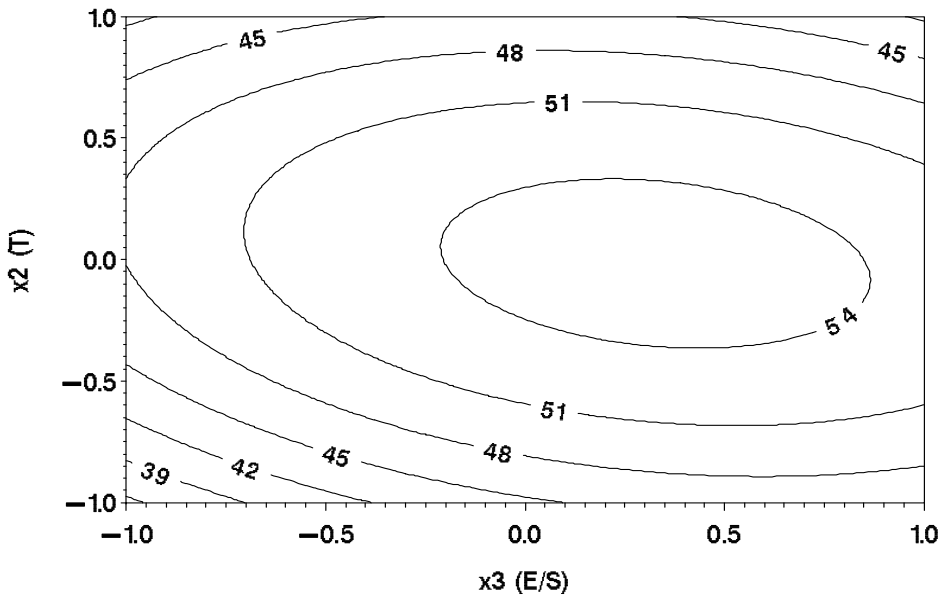


Figure 4.1 Pareto charts showing the significance of terms in models on ACE inhibition by Alcalase hydrolysates of peanut (a) and cowpea (b) flours.

Effects of three hydrolysis factors on ACE inhibitory activities of Alcalase hydrolysates of peanut and cowpea flours

Hypothesis tests for over-all effects of three factors proved the effects of hydrolysis temperature and E/S ratio are significant on ACE inhibitory activities of peanut and cowpea hydrolysates (Table 4.3),while p values for pH effect are higher than 0.05 over the range studied. The relationship between factors and response can be best understood by examining the contour plots generated by keeping one factor constant and plotting response as a function of two other factors. In Figure 4.2, an increase in ACE inhibition (%) is achieved by increasing hydrolysis temperature, up to certain levels, beyond which ACE inhibition (%) decreases. Such decrease in ACE inhibitory activities of peanut and cowpea protein hydrolysates over higher hydrolysis temperature may be explained by the increasing denaturation of the protease, reducing its biological activity (Diniz and Martin, 1996). Similar dependence between ACE inhibitory activity of protein hydrolysate and hydrolysis temperature has been observed by van der Ven *et al*. (2002) in the hydrolysis of whey protein by Corolase PP. A relative high E/S ratio is desirable to increase ACE inhibitory activity of Alcalase hydrolysates of peanut and cowpea flours. A slight decrease of ACE inhibition (%) under an extremely high E/S ratio range studied appears to indicate that inhibition of the hydrolysis enzyme occurs, including the possibility that the enzyme Alcalase hydrolyzes itself (Diniz and Martin, 1996). Canonical analysis revealed a maximum region for the models. The critical values of three hydrolysis factors and stationary points are concluded in Table 4.4. It is observed that the responses from stationary and central points are not different significantly.

a



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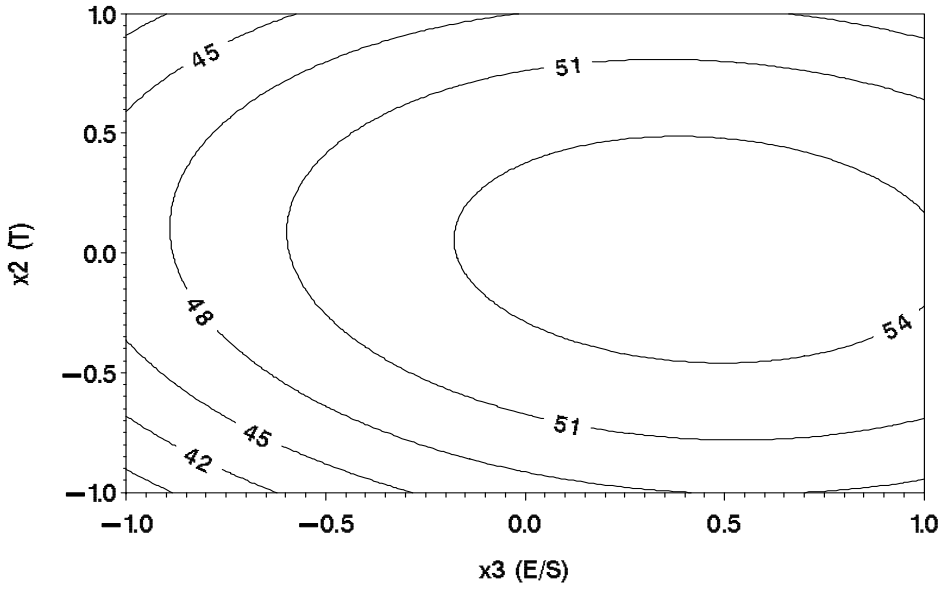

Figure 4.2 Contour plots showing the combined effect of hydrolysis temperature and enzyme/substrate ratio (E/S) on ACE inhibition (%) by Alcalase hydrolysates of peanut (a) and cowpea (b) flours. The factor of pH is set at its optimal value.

Table 4.4 Critical values of hydrolysis factors at stationary point.

	Critical value						
	Factor						Response
	Coded			Uncoded			
	x_1	x_2	x_3	pH	T	E/S	
Peanut	0.106965	-0.016162	0.326722	7.6	59.8	0.395	55.1
Cowpea	0.000497	0.014387	0.438109	7.5	60.2	0.284	55.6

CONCLUSION

The presented results show response surface methodology can be applied efficiently to define the effects of protein hydrolysis factors on ACE inhibitory activities of the resulting hydrolysates and thus optimize the hydrolysis conditions. The significance of hydrolysis factors depends on the ranges studies. When pH value is set from 6.5-8.5, ACE inhibition shows no significant sensitivity to pH change, whereas ACE inhibition is significantly influenced by temperature and E/S ratio over the ranges studied. ACE inhibition is a result of the inhibitory action of various peptides in the hydrolysate. This study proves that peanut and cowpea proteins are good sources of ACE inhibitory peptides. Further research is required to purify and characterize ACE inhibitory peptides from Alcalase hydrolysates of peanut and cowpea flours.

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CHAPTER 5

PURIFICATION, ACTIVITY AND SEQUENCE OF ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDES FROM ALCALASE HYDROLYSATES OF PEANUT AND COWPEA FLOURS[1]

[1]Cuie Guang, Robert D. Phillips, to be submitted to the J Agric. Food Chem.

ABSTRACT

Peanut and cowpea hydrolysates obtained after 6 h of digestion by Alcalase were used to isolate angiotensin I converting enzyme (ACE) inhibitory peptides. After centrifugation and ultrafiltration through a 0.2 μm nylon filter, the hydrolysate was filtered through the polyethersulphone membrane with a molecular weight cutoff (MWCO) of 10 kDa. The resulting permeate was then separated by primary reverse-phase high performance liquid chromatography (RP-HPLC). Eluate was divided into six major fractions according to eluation time. The fraction with eluting time 50-60 min showed the most potent ACE inhibition and was subjected to further purification by the secondary RP-HPLC. Four peaks were found to have strong ACE inhibitory activities and their IC_{50} values were determined. Peptides were identified by matrix assisted-laser desorption/ionization (MALDI), electrospray ionization (ESI) and MALDI tandem TOF-TOF (time-of-flight) mass spectrometer (MS/MS), as Lys-Ala-Phe-Arg from peanut and Phe-Phe from cowpea.

Key words: Angiotensin I converting enzyme, Peanut protein, Cowpea protein, ACE inhibitory peptides.

INTRODUCTION

In living organisms, endogenous peptides usually function through hormone-receptor interactions and signaling cascades. Bioactive peptides from food proteins also exert hormone-like regulatory activities in the human organism (Wang and Gonzalez de Mejia, 2005). Among them, those with antihypertensive effects have been extensively studied for different food proteins. It has been shown that short peptide sequences are potentially potent inhibitors of angiotensin I converting enzyme (ACE; EC 3.4.15.1), a dipeptidyl carboxypeptidase with a major role in the regulation of blood pressure (Yust *et al*., 2003).

ACE inhibitory peptides are usually separated from a hydrolysate mixture by various kinds of membrane-based separation and chromatography techniques. It has been reported that permeates of potato liquid fraction (Pihlanto *et al*., 2008) and soy protein hydrolysates (Wu and Ding, 2002; Chiang *et al*., 2006) from a 10 kDa membrane had no significant difference in ACE inhibitory activities when compared to their permeates from studied membranes with smaller molecular weight cut-offs (MWCOs), and therefore were selected for further purification. Reverse-phase high performance liquid chromatography (RP-HPLC) separates compounds according to their hydrophobic character and has been the most widely used to isolate ACE inhibitory fractions and peptide. Mass spectrometric techniques can provides molecular mass and amino acid sequence information. They are also effective in confirming the purity of peptides.

Peanut (*Arachis hypogeae*) is an important source of arginine-rich dietary protein as well as vitamins, fiber and other nutrients. Cowpea (*Vigna unguiculata*) is a starchy legume that is also an important source of dietary protein. Previous research has shown

peanut protein to be a potential source of ACE inhibitory peptides (Quist *et al*., 2009) and Alcalase hydrolysate of cowpea protein to cause ACE inhibition. A recent study showed that ACE inhibition began to plateau after peanut was hydrolyzed for 2.5 h and cowpea for 1 h, and that inhibitory activity remained constant thereafter. The 6-hour hydrolysates resulting from Alcalase hydrolysis of these protein sources were used as starting materials for further purification. The aim of this study was therefore to purify ACE inhibitory peptides from Alcalase hydrolysates of peanut and cowpea flours by membrane filtration and RP-HPLC and sequence them by mass spectrometry.

MATERIALS AND METHODS

Assay of ACE inhibitory activity

The microplate kinetic assay of ACE inhibition was the same as described in Chapter 3.

A plot of log ((100-ACE inhibition (%))/ACE inhibition (%)) versus log (peptide concentration, μg/ml) was generated using different concentrations of samples. The IC_{50} value was expressed in terms of μg/ml, defined as the concentration of inhibitor which gave 50% ACE inhibition, and calculated using the linear regression equations of the curves. Spectrophotometric assay to determine peptide concentrations were performed on Thermo Spectronic Spectrophotometer (Genesys 6, Thermo Electron Co., Madison, WI) at 215 nm and 225nm. Peptides concentration (μg/ml) was calculated from UV absorbance of properly diluted solutions using the formula

$$\text{Concentration} = (A215\text{-}A225) \times 144$$

Purification of ACE inhibitory peptides

The 6-hour hydrolysate from 0.2 μm Millipore nylon filter was filtered through the Biomax polyethersulphone membrane (Millipore Co., Billerica, MA) with a MWCO of 10 kDa in Labscale TFF System (Millipore Co., Billerica, MA). The resulting permeate (1000 μl) was injected and separated by primary reverse-phase high performance liquid chromatography (RP-HPLC) system comprised of a Jupiter 4u Proteo 90A C12 preparative reversed-phase column (250×21.10 mm, Phenomenex, Inc., Torrance, CA), a Waters 2690 Separations Module and a Waters 996 photodiode array detector (Waters Co., Milford, MA) recording absorbance from 210 to 300 nm. Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in filtrated deionized water, and solvent B was 0.1% (v/v) TFA in acetonitrile. A linear gradient from 0% to 75% of solvent B within 75 min was applied at a flow rate of 3 ml/min. Eluting peaks were collected as six major fractions using a Waters Fraction Collector III (Waters Co., Milford, MA), pooled and lyophilized using a Genesis SQ freeze dryer (VirTis Co., Gardiner, NY) with a dry-ice secondary trap in the vacuum line. The most potent fraction (100 μl) was injected and further isolated by secondary RP-HPLC system with a Jupiter 4u Proteo 90A C12 column (250×10 mm, Phenomenex, Inc., Torrance, CA). Elution was achieved by a linear gradient from 35% to 42.5% solvent B within 30 min at a flow rate of 1 ml/min. Individual peaks were collected, pooled and lyophilized.

Mass spectrometry and sequences of ACE inhibitory peptides

The molecular mass of the purified ACE inhibitory peptides was determined using a Perkin Elmer Sciex API I plus quadrupole mass spectrometer (Foster City, CA)

or an Autoflex matrix-assisted laser desorption and ionization time-of-flight mass spectrometer (MALDI-TOF, Brucker Daltonics Inc., Billerica, MA). For API, the lyophilized sample was first dissolved in 50% acetonitrile containing 0.1% TFA and 20 uL of the sample solution was injected and pumped through a narrow, stainless steel capillary. A high voltage of 4 kV was applied to the tip of the capillary, which is situated within the ionization source of the mass spectrometer, and a stream of nebulizing gas was also introduced. As a consequence, the sample emerging from the tip was dispersed into highly charged droplets. These droplets were then evaporated at the interface by the drying gas. Eventually charged sample ions, free from solvent, were released from the droplets and detected by the analyzer. API (atmospheric pressure ionization) is similar to electrospray ionization (ESI) and these two terms are usually used interchangeably. For MALDI, 1µl of the sample solution was mixed with 1 µl of matrix solution (α-cyano-4-hydroxycinnamic acid saturated in the mixture of acetonitrile and 0.1% TFA solution in a volume ratio of 1:1) and let dry on a stainless steel MALDI target. The dried sample was introduced into the mass spectrometer where a nitrogen laser (337 nm wave length) was fired to desorb and ionize the sample. The time-of flight analyzer separated ions according to their m/z ratios by measuring the time it took for ions to travel through a field free region. The m/z ratio of the mass spectrometer was calibrated with angiotensin II digested by trypsin in external and internal calibration modes. The peptide sequence was determined by a MALDI tandem TOF-TOF mass spectrometer (MS/MS). Sample preparation was the same as that for molecular mass determination using MALDI-TOF. The samples were measured in the reflection and positive ionization mode. All tandem

mass spectra were acquired by an Applied Biosystems 4700 Proteomics Analyzer (Foster City, CA).

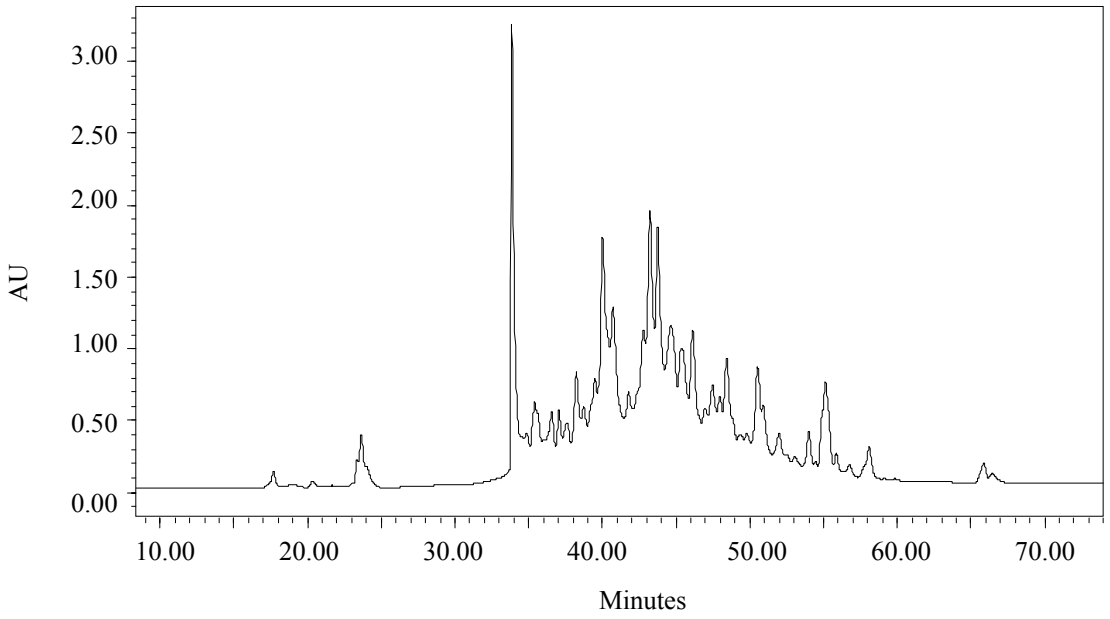
RESULTS AND DISCUSSION

Purification and activity of ACE inhibitory peptides from Alcalase hydolysates of peanut and cowpea flours

ACE inhibition began to plateau after peanut was hydrolyzed for 2.5 h and cowpea for 1 h. The hydrolysates obtained after 6 h of digestion by Alcalase were used to isolate ACE inhibitory peptides. After centrifugation and ultrafiltration through a 0.2 μm Millipore nylon filter (Bellerica, MA), hydrolysates had IC_{50} values of 134.4 and 123.6μg/ml for peanut and cowpea, respectively, which are lower than that for Alcalase hydrolysates of defatted wheat germ (670 μg/ml) (Matsui *et al*., 1999), isolated soy protein (668 μg/ml) (Chiang, *et al*., 2006), mung bean protein isolate (640 μg/ml) (Li *et al*., 2006), defatted soy meal (340 μg/ml) (Wu and Ding, 2002), corn gluten (197 μg/ml) (Yang *et al*., 2007), and rice protein (140 μg/ml) (Li *et al*., 2007), but is higher than that for Alcalase hydrolysate of potato hydrolysates (18-86 μg/ml) (Pihlanto *et al*., 2008). Therefore, peanut and cowpea protein hydrolysates obtained by Alcalase digestion may be considered as functional foods.

The hydrolysates were filtered through a polyethersulphone membrane with a MWCO of 10 kDa. The resulting permeates had IC_{50} values of 85.8 and 95.7 μg/ml (Figure 5.3) for peanut and cowpea, respectively, which are lower than that for a 10 kDa soy permeate of Alcalase hydrolysate of Wu and Ding (180 mg/ml) (2002) but higher

a



b

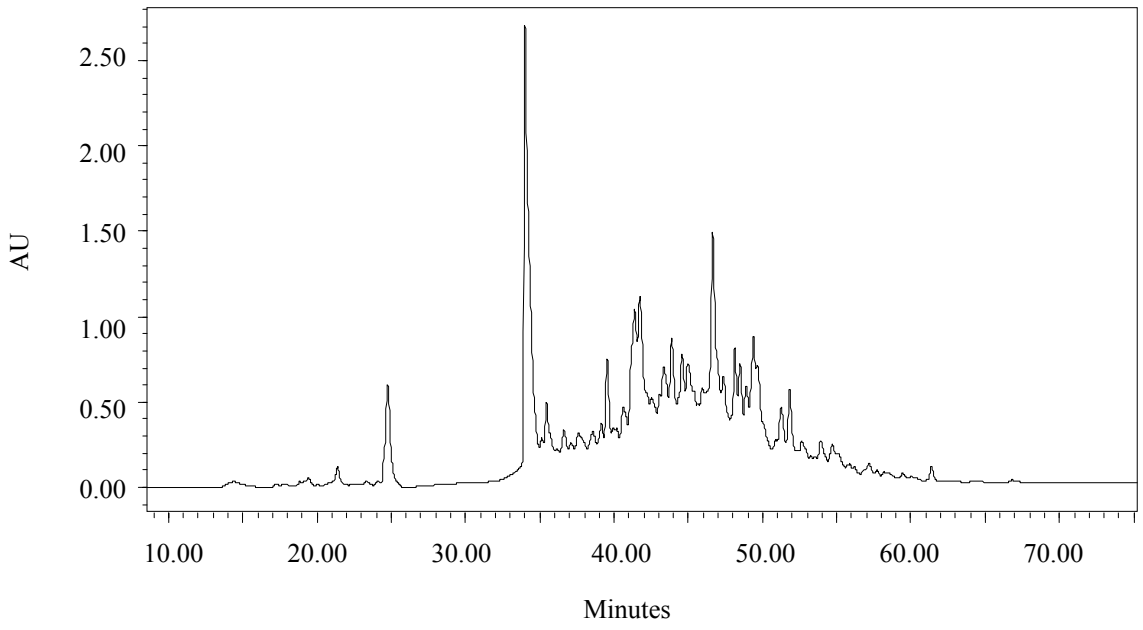
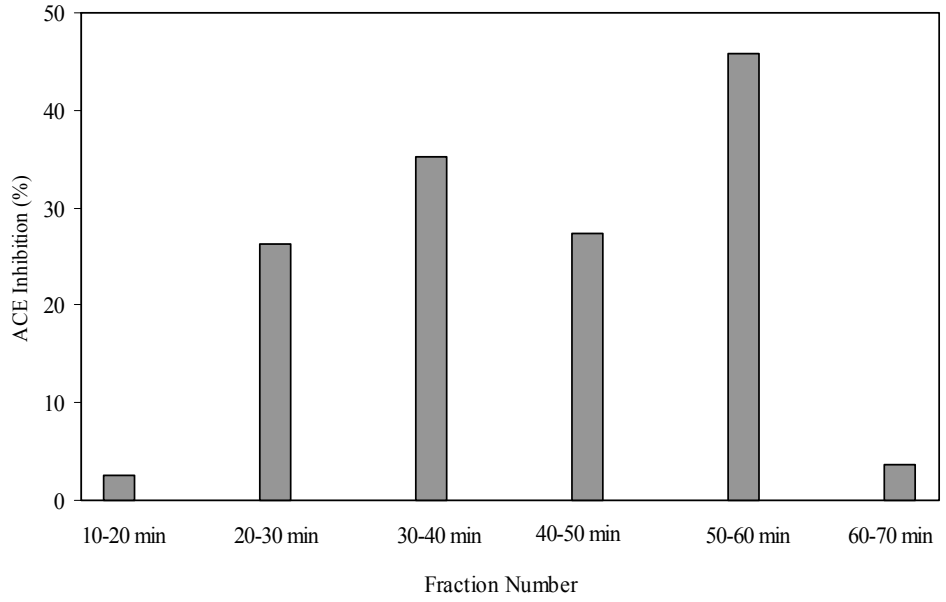


Figure 5.1 RP-HPLC profiles of the 10-kDa membrane permeates of peanut (a) and cowpea (b) hydrolysates at 215 nm.

a



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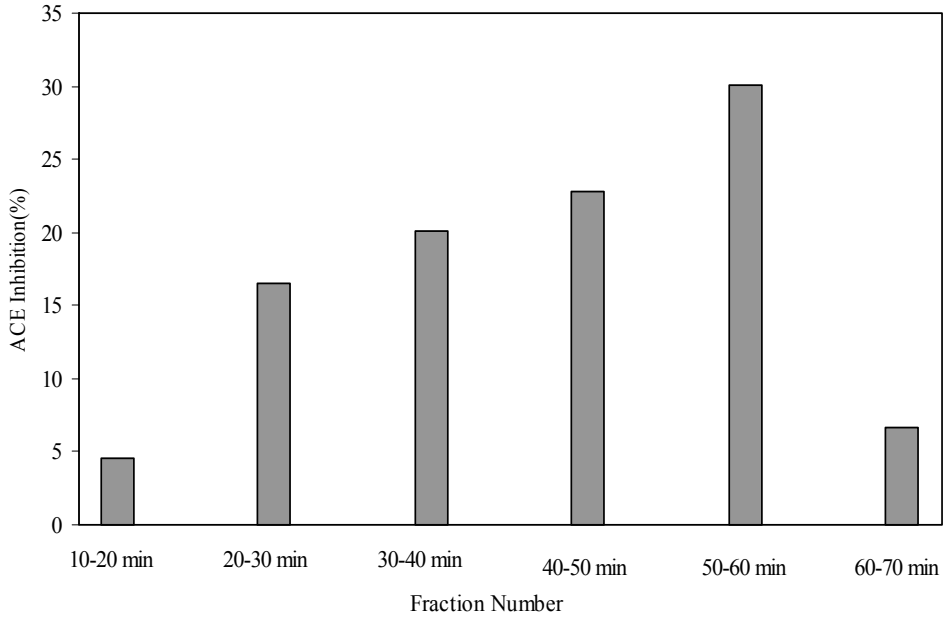
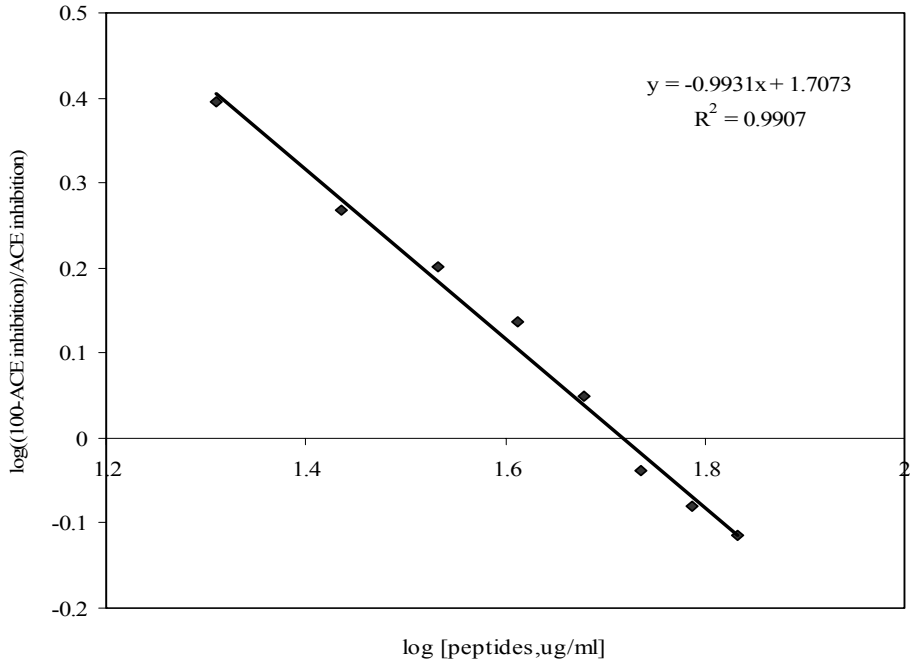


Figure 5.2 ACE inhibitions of six major fractions after primary RP-HPLC. ACE inhibitions (%) were measured at peptide concentration 45.5 μg/ml for peanut fractions (a) and 32.7 μg/ml for cowpea fractions (b).

a



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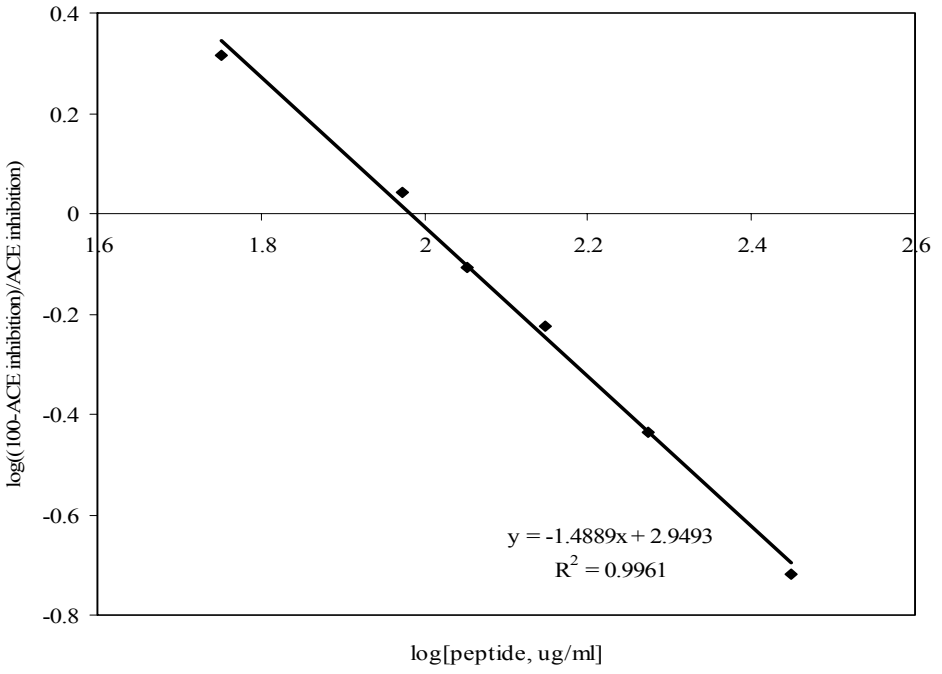
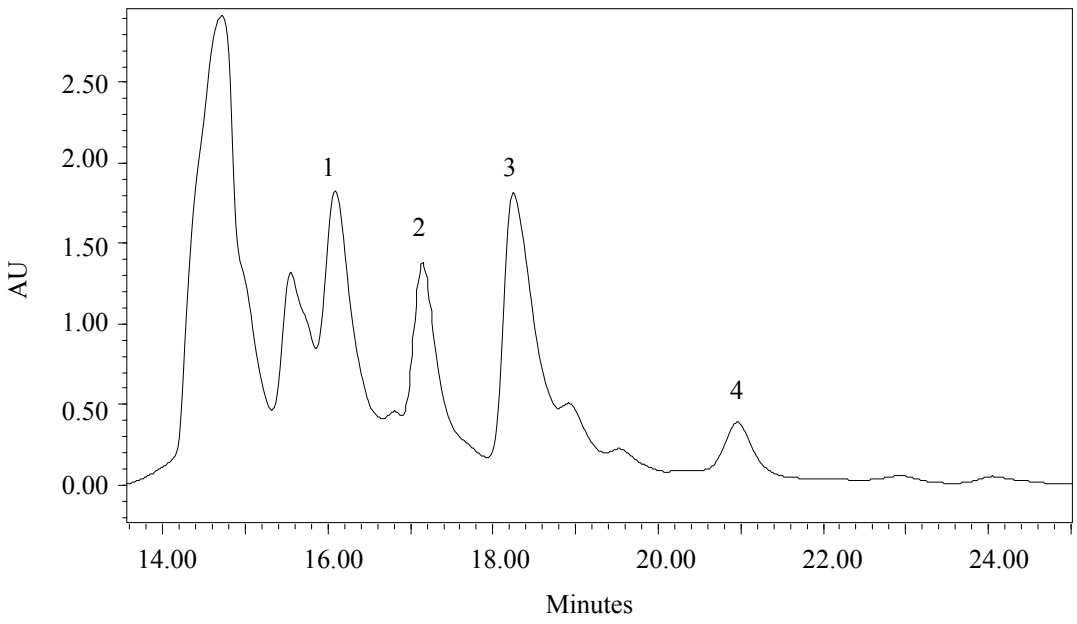


Figure 5.3 Determination of IC_{50} values for 50-60 min peanut fraction (a) and cowpea membrane permeate (b).

a



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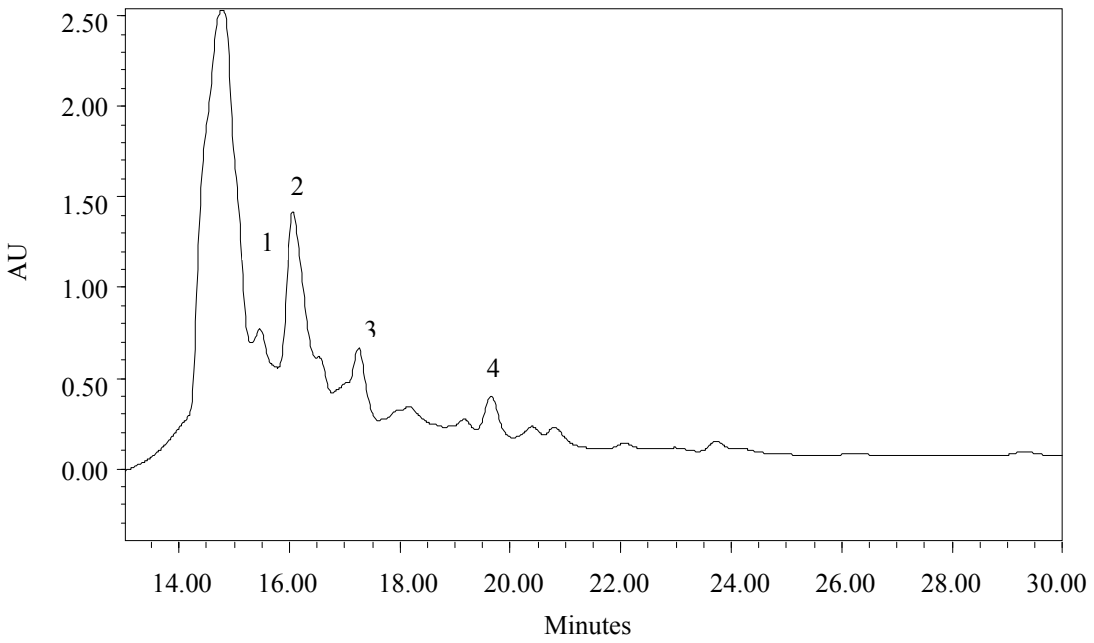


Figure 5.4 RP-HPLC profiles of 50-60 min peanut (a) and cowpea (b) fractions at 215 nm.

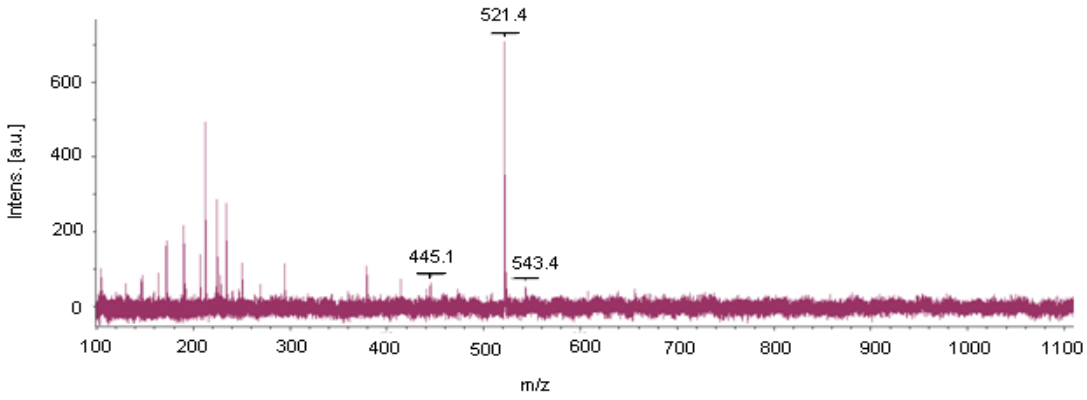
Table 5.1 IC_{50} values of potent peaks after the secondary RP-HPLC.

Peaks	Peanut	Cowpea
	IC_{50} (μg/ml)	
1	33.1	22.0
2	27.1	25.0
3	24.5	31.1
4	16.9	28.4

Table 5.2 Effects of purification steps on IC_{50} values

Purification step	Peanut	Cowpea
	IC_{50} (μg/ml)	
Hydrolysate	134.4	123.6
Membrane	85.8	95.7
Primary RP-HPLC	52.4	58.5
Secondary RP-HPLC	16.9	22.0

a



b

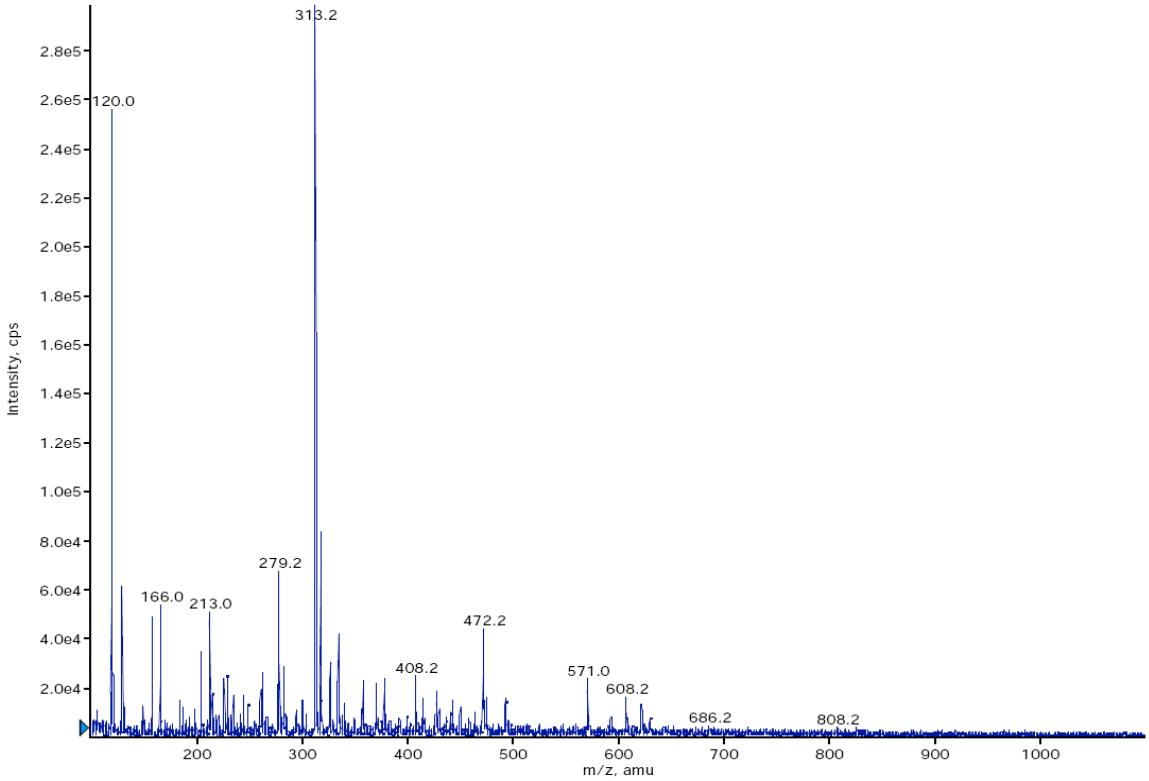
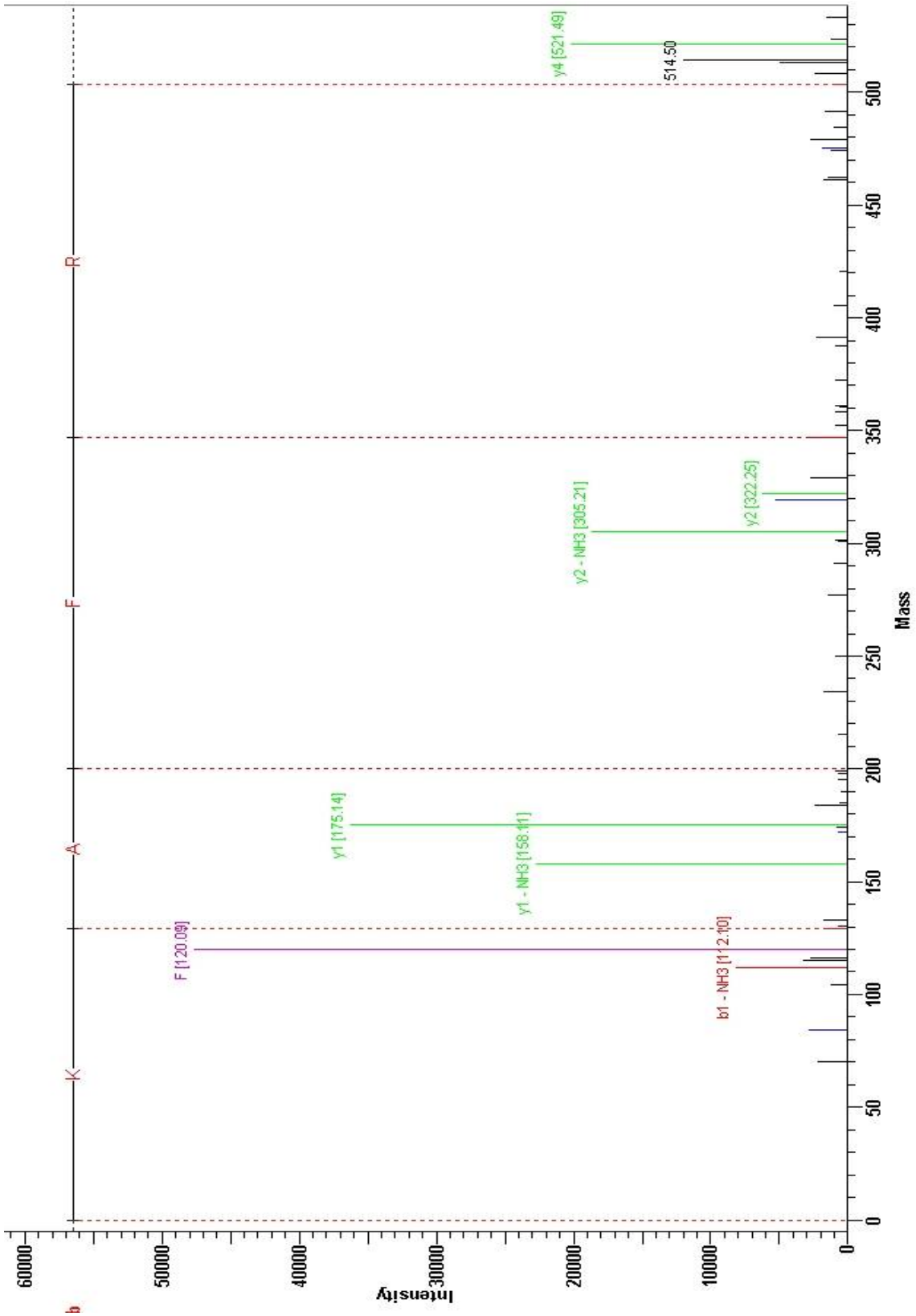


Figure 5.5. MALDI mass spectrum of peanut peak 4 (a) and ESI mass spectrum of cowpea peak 1 (b). The x-axis shows the mass to charge ratio (m/z) and the y-axis shows the intensity in arbitrary units (a.u.) (a) and relative intensity with counts proportional to signal intensity (cps) (b).

a


y4 [521.49]
514.50
y2 - NH3 [305.21]
y2 [322.25]
y1 [175.14]
y1 - NH3 [158.11]
F [120.09]
b1 - NH3 [112.10]
K
A
F
R
Intensity
Mass
0
10000
20000
30000
40000
50000
60000
0
50
100
150
200
250
300
350
400
450
500

b

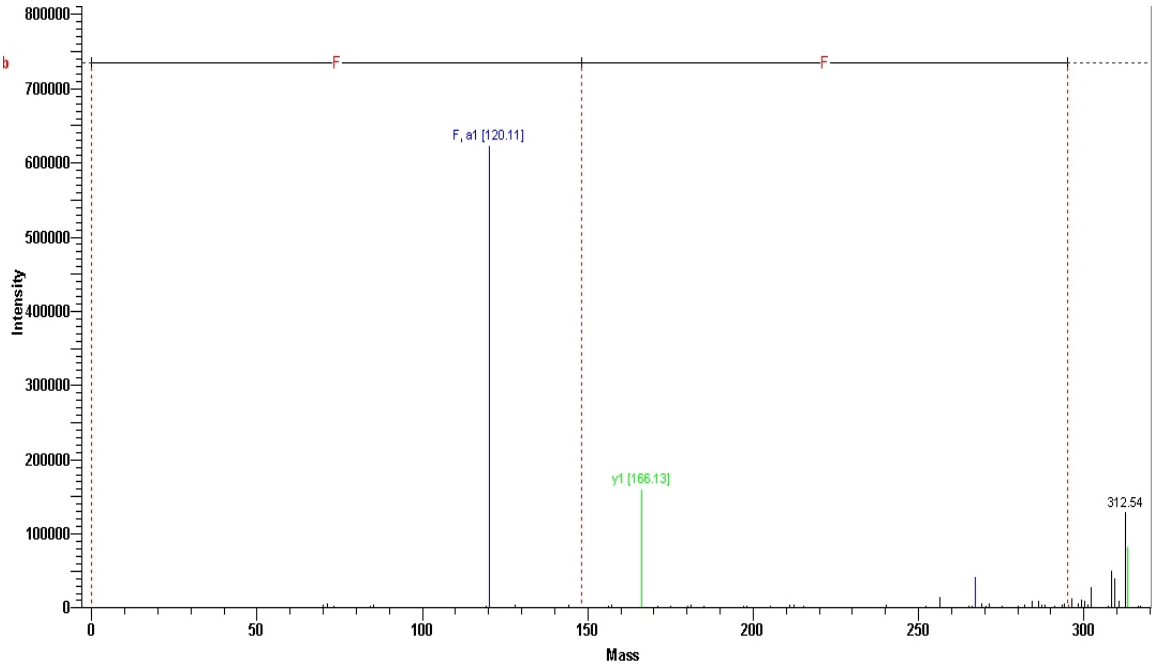


Figure 5.6 Tandem mass spectrometry (MS/MS) spectra of ion m/z 521.4 from peanut (a) and ion m/z 313.2 from cowpea (b). Dotted lines represent identified b ions of ACE inhibitors.

than that for another 10 kDa soy permeate of Alcalase hydrolysate of Chiang *et al*.(78 mg/ml) (2006).

The resulting permeates were then separated by primary RP-HPLC (Figure 5.1). The eluates were divided into six major fractions according to eluation time. The eluates with eluting time 50-60 min were found to have the most potent ACE inhibition (Figure 5.2) with IC_{50} values being 52.4 (Figure 5.3) and 58.5μg/ml for peanut and cowpea, respectively, and were used for further purification. It should be pointed out that all other fractions also have ACE inhibitory activities though their capabilities are lower than that of 50-60 min fractions. After isolation by secondary RP-HPLC, four peaks had the strong

ACE inhibition (Figure 5. 4). Their IC_{50} values are summarized in Table 5.1. Table 5.2 shows how the IC_{50} decreased step by step with the purification progress, which indicates that the isolation procedure is effective. In order to identify peptide sequences, the strong peaks from secondary RP-HPLC were first subjected to MALDI and ESI to confirm the purity and molecular mass. The mass spectra of peanut peak 4 and cowpea peak 1 are shown in Figure 5.5. The singly charged ions with m/z 521.4 and 313.2 are found to be the most dominant for peanut peak 4 and cowpea peak 1, respectively. Two trace ions with m/z 445.1 and 543.4 in Figure 5.5 (a) obtained by MALDI didn't appear in the mass spectra obtained by ESI and MS/MS and only ion with m/z 521.4 was present in all three mass spectra and was always the most dominant. In Figure 5.5 (b), the ion with m/z 120.0 was not a peptide though it shows strong intensity. Similarly, other ions in ESI mass spectrum didn't appear in mass spectra from MALDI and MS/MS. So only ions at m/z 521.4 and 313.2 were subjected to the following fragmentation.

The tandem mass spectra of the two parent ions and their amino acid sequences are shown in Figure 5.6. A major fragment ion was observed at m/z 120.1 in both tandem mass spectra and it is the immonium ion of phenylalanine. It is produced as a secondary fragmentation (a combination of a y and a-type cleavage) of the amide bond during collision induced dissociation and is a strong predictor of corresponding amino acid present in the sequence. The presence of arginine in the C-terminus of peptide KAFR favored the appearance of y-type fragmentation ions (Li *et al*., 2006) that are very common and easily identifiable. Dotted lines in tandem mass spectra represent b ions and thus ACE inhibitory peptides are identified as KAFR and FF for peanut and cowpea, respectively.

ACE inhibition studies with varying structure of dipeptides show that C-terminal phenylalanine contributes to peptide binding and ACE inhibitory activity (Cheung et al., 1980). Although ACE appears to have a preference for a substrate or a competitive inhibitor containing hydrophobic (aromatic or branched side-chains) amino acids in the C-terminal tripeptide, structure-activity data suggest that a C-terminal arginine with a positive charge on the ε-amino group seems to contribute substantially to ACE inhibitory potency, which indicates a possible interaction between the inhibitor and an anionic binding site of ACE that is different from the catalytic site. The removal of the arginine residue at the C-terminus can lead to essentially inactive peptide analogues (FitzGerald and Meisel, 2000; Murray and FitzGerald, 2007). The amino acid arginine also seems to contribute to ACE inhibitory potency when situated at a non-C-terminal position (Rohrbach et al., 1981; Kumada *et al*., 2007). For the relatively long peptides, the steric structure adopted in specific environment affects ACE inhibitory activities (Pripp et al., 2004).

CONCLUSION

Peanut and cowpea have been consumed worldwide due to their nutrition and availability. The present research has shown that peanut and cowpea proteins are also good sources of ACE inhibitory peptides. The product from each purification step is comparable in ACE inhibitory activity with products from other sources and may be used as a health enhancing ingredient in functional foods. Purification results in up to an 8 fold increase in ACE inhibitory activity over the crude hydrolysate, which indicates the effectiveness of isolation procedure. Peptides KAFR and FF are more or less consistent

with the structure requirements proposed for ACE inhibitory peptides. Further research is required to confirm their *in vivo* activities.

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CHAPTER 6

APPLICATION OF FOUR SETS OF AMINO ACID DESCRIPTOR SCALES TO THE DESCRIPTION OF QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIP OF ACE INHIBITORY DIPEPTIDES[1]

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[1]Cuie Guang, Robert D. Phillips, to be submitted to the Journal of Biomedicine and Biotechnology.

ABSTRACT

Activity of angiotensin I converting enzyme (ACE) inhibitory dipeptide is a function of molecular structure and their relationship can be modeled using partial least square (PLS) regression. In this study, four sets of amino acid descriptor scales from two separate labs were used to investigate their performance in quantitative structure-activity relationship (QSAR) study. The final model based on factor analysis scales of generalized amino acid information (FASGAI) were selected to predict activities for ACE inhibitory dipeptides including dipeptide FF previously isolated from Alcalase hydrolysate of cowpea. The preferred structure was also proposed. A potent ACE inhibitory dipeptide should have a large and hydrophobic amino acid such as Trp and Phe at the C-terminus and an amino acid that is nonpolar such as Val, Leu and Ile or possibly positively charged such as Arg and Lys at the N-terminus.

Key words: ACE inhibitory dipeptides, Quantitative structure-activity relationship, Partial least square regression, Amino acid descriptors.

INTRODUCTION

Peptides play important roles in living organisms and thus attract considerable interest in pharmacology and nutrition. Following the isolation of bioactive peptides, the systhesis of peptides and analogues to achieve therapeutic or nutritional purposes has been made possible (Sandberg *et al*., 1998). The biological activity of a peptide depends on the properties of compositional amino acids and any change in amino acids would probably affect its potency. Therefore, it is of great use to model the relationship between structure and activity. Quantitative structure-activity relationship (QSAR) study not only predicts the activity of new peptides but also indicates the preferred structure for active peptides.

Amino acid descriptors are usually used to describe the structures of peptides. The first amino acid descriptors were derived by Sneath (1966) from physicochemical semiqualitative data for 20 coded amino acids and were used in a quantitative sequence-activity model (QSAM) analysis of oxytocin-vasopressine analogues. Hellberg *et al*. (1987) extended the multivariate approach of Sneath to continuous amino acid properties and expanded the property matrix to 29 physicochemical variables of 20 coded amino acids. The multiproperty matrix was extracted by principle component analysis (PCA) to give three scales z_1, z_2, and z_3 (Appendix A). The three resulting principle components were linear combination of primary parameters and were tentatively interpreted as related to hydrophilicity (z_1), bulk (z_2), and electronic properties (z_3) (Table 6.1). The z scales have shown the usefulness in modeling and predicting biological activities of oxytocins, pseudopeptides, pepstatins, and bradykinin potentiating peptides. By using 26 descriptors, Sandberg et al. (1998) expanded the 3-z scales to the 5-z scales to encompass

Table 6.1 Interpretation of descriptor scales for amino acids.

3-z scale	z_1: hydrophobicity	VHSE [a]	VHSE1 (v1), VHSE2 (v2): hydrophobicity
	z_2: bulk		VHSE3 (v3), VHSE4 (v4): steric properties
	z_3: electronic properties		VHSE5 (v5)-VHSE8 (v8): electronic properties
5-z scale	z1: lipophilicity	FASGAI[b]	factor 1 (f1): hydrophobicity
	z_2: steric bulk, polarizability		factor 2 (f2): alpha and turn properties
	z_3: polarity		factor 3 (f3): bulk
	z_4: electronegativity, heat of formation		factor 4 (f4): composition
	z_5: electrophilicity, hardness, etc		factor 5 (f5): local flexibility
			factor 6 (f6): electronic properties

[a] principal components score vectors of hydrophobic, steric, and electronic properties; [b] factor analysis scales of generalized amino acid information.

20 coded and 67 noncoded amino acids (Appendix B). In the new 5-z scales, the first three were interpreted as the same as in the 3-z scales. The fourth and fifth scales, z_4 and z_5, however, were more difficult to interpret. They were related to electronegativity, heat of formation and electrophilicity, hardness, and NMR at pD=1 and 7 (Table 6.1). The 5-z scales revealed validity in the QSAM analysis of elastase substrates and neurotensin analogues. The two sets of scales were from the same lab. Recently, two new sets of amino acid descriptors from another lab were developed and were called principle components score vectors of hydrophobic, steric, and electronic properties (VHSE) (Mei *et al*., 2005) (Appendix C) and factor analysis scales of generalized amino acid information (FASGAI) (Liang *et al*., 2009) (Appendix D). VHSE descriptors were derived separately from the PCA of three independent families containing 18 hydrophobic properties, 17 steric properties, and 15 electronic properties, respectively.

FASGAI descriptors involved hydrophobicity, alpha and turn propensities, bulky properties, compositional characteristics, local flexibility and electronic properties (Table 6.1).

ACE (EC 3.4.15.1) is a monomeric, type I membrane-bound glycoprotein that catalyzes the formation of vasoconstrictor, angiotensin II and the inactivation of vasodilator, bradykinin. The influences of ACE on blood pressure make it an ideal target clinically and nutritionally in the treatment of hypertension. Some peptide analogues have been developed as clinical drugs and a number of ACE inhibitory peptides have also isolated from food sources (Murray and FitzGerald, 2007). The previous research has isolated a dipeptide Phe-Phe from Alcalase hydrolysate of cowpea flours. In this study, the validity of these four sets of amino acids descriptors in modeling the relationship of structure and activity of ACE inhibitory dipeptides was investigated; the activities of dipeptides were predicted; and the preferred structure was proposed for ACE inhibitory dipeptides.

MATERIALS AND METHODS

Collection of ACE inhibitory dipeptides

The primary structure of ACE inhibitory dipeptides and their inhibitory activity expressed as peptide concentration (μM) required to inhibit 50% of ACE activity (IC_{50}) were collected from previously published articles (Table 6.2). An individual peptide may have several reported IC_{50} values from different sources. In this case, each value was included. Modeling work revealed that a logarithmic transformation of IC_{50} values could

Table 6.2 The observed (Obsv) and predicted (Pred) activities (log IC_{50}) of ACE inhibitory dipeptides.

No.	Ptd[a]	Obsv	Pred[b]	Resd[c]	No.	Ptd	Obsv	Pred	Resd
1	RP	-0.42			51	LY	1.59	1.23	0.36
2	MF	-0.04	1.28	-1.32	52	FY	1.63	1.31	0.32
3	FL	0.12			53	VF	1.64	1.23	0.41
4	VW	0.15	0.67	-0.52	54	MF	1.65	1.28	0.37
5	FE	0.16			55	NF	1.67	1.83	-0.16
6	VW	0.20	0.67	-0.47	56	LW	1.70	0.71	0.99
7	KW	0.21	0.63	-0.42	57	RY	1.71	0.90	0.81
8	KY	0.21	1.15	-0.94	58	VF	1.72	1.23	0.49
9	IW	0.30	0.72	-0.42	59	WL	1.81	2.24	-0.43
10	CF	0.30	1.54	-1.24	60	SY	1.82	1.73	0.09
11	IY	0.32	1.23	-0.91	61	IF	1.85	1.28	0.57
12	IY	0.36	1.23	-0.87	62	GY	1.86	2.22	-0.36
13	VW	0.40	0.67	-0.27	63	YL	1.91	2.30	-0.39
14	IY	0.43	1.23	-0.80	64	AY	1.94	1.50	0.44
15	EY	0.43	1.46	-1.03	65	WM	1.98	1.68	0.30
16	IY	0.57	1.23	-0.66	66	AY	2.00	1.50	0.50
17	FY	0.57	1.31	-0.74	67	YL	2.09	2.30	-0.21
18	VL	0.59	2.05	-1.46	68	LF	2.10	1.27	0.83
19	IF	0.62	1.28	-0.66	69	IP	2.11	2.43	0.32
20	IW	0.67	0.72	0.05	70	SF	2.11	1.77	0.34
21	LY	0.81	1.23	-0.42	71	IA	2.18	2.88	-0.70
22	VY	0.85	1.19	-0.34	72	RP	2.26	2.10	0.16
23	VF	0.96	1.23	-0.27	73	AF	2.28	1.54	0.74
24	AW	1.00	0.98	0.02	74	MY	2.29	1.24	1.05
25	VY	1.00	1.19	-0.19	75	GY	2.32	2.22	0.10
26	MW	1.00	0.72	0.28	76	AP	2.36	2.70	-0.34
27	YW	1.02	0.93	0.09	77	RF	2.36	0.94	1.42
28	IY	1.02	1.23	-0.21	78	GP	2.40	3.42	1.02
29	RY	1.02	0.90	-0.12	79	GY	2.42	2.22	0.20
30	DG	1.09			80	WA	2.44	3.03	-0.59
31	VK	1.11	2.46	-1.35	81	TP	2.46	2.71	-0.25
32	KY	1.11	1.15	-0.04	82	FP	2.50	2.51	-0.01
33	AY	1.15	1.50	-0.35	83	PL	2.53		
34	AF	1.18	1.54	-0.36	84	LF	2.54	1.27	1.27
35	RW	1.20	0.38	0.82	85	GP	2.56	3.42	-0.86
36	VY	1.20	1.19	0.01	86	DF	2.56	1.94	0.62
37	YL	1.21	2.30	-1.09	87	VP	2.62	2.40	0.23
38	LW	1.24	0.71	0.53	88	GP	2.65	3.42	-0.77
39	TF	1.25	1.55	-0.30	89	FC	2.68	1.90	0.79
40	RP	1.32	2.10	-0.78	90	YV	2.76	2.27	0.49
41	KP	1.34	2.35	-1.01	91	VP	2.76	2.39	0.37
42	VY	1.34	1.19	0.15	92	DM	2.78	2.19	0.59
43	FY	1.40	1.31	0.09	93	YQ	2.80	2.48	0.32
44	HY	1.41	1.36	0.05	94	GF	2.80	2.26	0.54
45	KF	1.45	1.19	0.26	95	IR	2.84	2.18	0.66
46	AP	1.46	2.70	-1.24	96	YP	2.86	2.64	0.22
47	GW	1.48	1.70	-0.22	97	IF	2.97		
48	WL	1.48	2.24	-0.76	98	VG	3.04	3.15	-0.11
49	NY	1.51	1.78	-0.27	99	GI	3.08	2.61	0.47
50	VY	1.55	1.19	0.36	100	IG	3.08	3.19	-0.11

Table 6.2 (continued)

No.	Ptd	Obsv	Pred	Resd	No.	Ptd	Obsv	Pred	Resd
101	EP	3.08	2.66	0.42	117	GS	3.58	3.84	-0.26
102	RG	3.08	2.86	0.22	118	GV	3.66	3.05	0.61
103	GI	3.11	2.61	0.50	119	MG	3.68	3.20	0.48
104	GM	3.15	2.51	0.64	120	GK	3.73	3.49	0.24
105	YG	3.18	3.40	-0.22	121	GE	3.73	3.69	0.04
106	GA	3.30	3.86	0.56	122	GT	3.76	3.47	0.29
107	DL	3.30	2.76	0.54	123	WG	3.77	3.35	0.42
108	YG	3.30	3.40	-0.10	124	HG	3.80	3.32	0.48
109	NP	3.36	2.98	0.38	125	GQ	3.85	3.25	0.60
110	RL	3.39			126	GG	3.86	4.18	-0.32
111	GL	3.40	3.08	0.32	127	QG	3.87	3.30	0.57
112	AG	3.40	3.46	-0.06	128	SG	3.93	3.69	0.24
113	GH	3.49	2.80	0.69	129	LG	3.94	3.19	0.75
114	GR	3.50	3.16	0.34	130	GD	3.96	3.65	0.31
115	KG	3.51	3.11	0.40	131	TG	4.00	3.47	0.53
116	FG	3.57	3.27	0.30	132	FF		1.35	

[a] peptides; [b] peptides without predicted responses were outliers; [c] residues.

improve the model (Pripp *et al*., 2004) so in this study dependent variable was expressed as log IC_{50}.

Partial least square modeling

Partial least square (PLS) is widely used in chemometrics for QSAR study to describe how biological activities are influenced by structural variations in chemical compounds (Nash *et al*., 2005). PLS extracts successive linear combinations of the predictors. Extracted factors that account for predictor variation well are well represented by the observed value of the predictors, and extracted factors that explain response variation well provide good predictive models for new responses. PLS can balance the two objectives of explaining predictor variation and explaining response varation. PLS regression algorithm consists of outer relations (X and Y matrices of the predictors and responses) and an inner relation linking the two matrices (equation 3):

$$X= TP+E \qquad (1)$$

$$Y=UQ+F \quad (2)$$

$$U=BT \quad (3)$$

Where T is X-scores, P is X-loadings, E is X-residuals; and U is Y-scores, Q is Y-loadings, F is Y-residuals (SAS Institute Inc., Cary, NC). The T and U latent variables are correlated by the inner relation, which results in the estimation of Y from X (Mei et al., 2005). More detailed explanations for PLS regression are provided by Wold et al. (2001) PLS regression analysis between amino acid descriptors (predictor, X) and log IC_{50} (response, Y) was performed with SAS 9.1 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

The initial PLS models of ACE inhibitory dipeptides

The initial analysis based on four different sets of amino acid descriptors is summarized in Table 6.3. PLS extracted one significant factor for all four sets of amino acid desciptors using the cross validation method. The fitted models were tested and the predicted values were compared to that of observed using root mean PRESS (predictive residual sum of square). The lower the value of root mean PRESS, the better of the model (Nash and Chaloud, 2002). The PLS factor accounted for 23.1-26.7% and 52.6-58% of the variation for the predictor and ACE inhibitory activity data sets, respectively. The initial model based on VHSE scales explained higher percent of the variation in ACE inhibitory activity data than that based on other three scales.

Y-score and X-score values for the first factor showed high correlation and there was no particular clustering pattern in these points(Figure 6.1). But several observations showed a much worse fit than the others in the score plot. Peptides 1 (RP), 3 (FL), 5 (FE),

Table 6.3 The initial PLS models for ACE inhibitory dipeptides.

	Extracted factors	Root mean PRESS	Variation explained by PLS factors		Coefficient		VIP	
			independent	dependent	N	C	N	C
3-z scale	1	0.7637	26.7%	55.3%	z_1: 0.1925	0.3245	0.8027	1.3532
					z_2: *-0.1405*	-0.4020	*0.5856*	1.6763
					z_3: 0.1552	*-0.0521*	0.6470	*0.2172*
5-z scale	1	0.7768	24.1%	52.6%	z_1: 0.1196	0.2501	0.8088	1.6910
					z_2: *-0.0615*	-0.2789	*0.4158*	1.8858
					z_3: 0.1081	*-0.0618*	0.7312	*0.4177*
					z_4: *-0.0402*	-0.1645	*0.2717*	1.1123
					z_5: *-0.0270*	0.1525	*0.1827*	1.0314
FASGAI	1	0.7762	23.1%	53%	f1: *-0.0854*	-0.1266	*0.6766*	1.0031
					f2: -0.1797	*-0.0734*	1.4244	*0.5814*
					f3: -0.1128	-0.2536	0.8941	2.0098
					f4: *0.0249*	0.1646	*0.1971*	1.3041
					f5: *-0.0979*	*0.0255*	*0.7760*	*0.2020*
					f6: -0.1330	*-0.0783*	1.0542	*0.6203*
VHSE	1	0.7232	25.3%	58%	v1: *-0.0477*	-0.1234	*0.5037*	1.3035
					v2: *-0.0370*	-0.1682	*0.3912*	1.7768
					v3: -0.0903	-0.1609	0.9535	1.7000
					v4: 0.1090	*0.0308*	1.1515	*0.3256*
					v5: *-0.0641*	-0.1058	*0.6766*	1.1175
					v6: *-0.0014*	*-0.0715*	*0.0151*	*0.7552*
					v7: *0.0707*	0.1458	*0.7468*	1.5401
					v8: *-0.0725*	*0.0093*	*0.7663*	*0.0984*

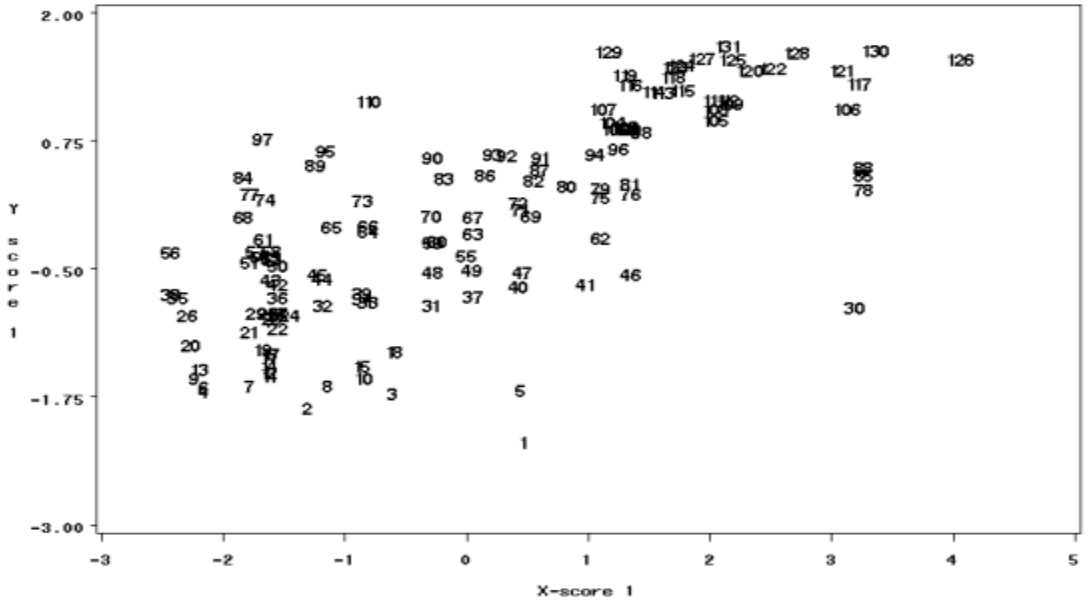

Figure 6.1 Plot of X- and Y-scores for factor 1 for each of observation from initial PLS model of ACE inhibitory dipeptides based on FASGAI scales.

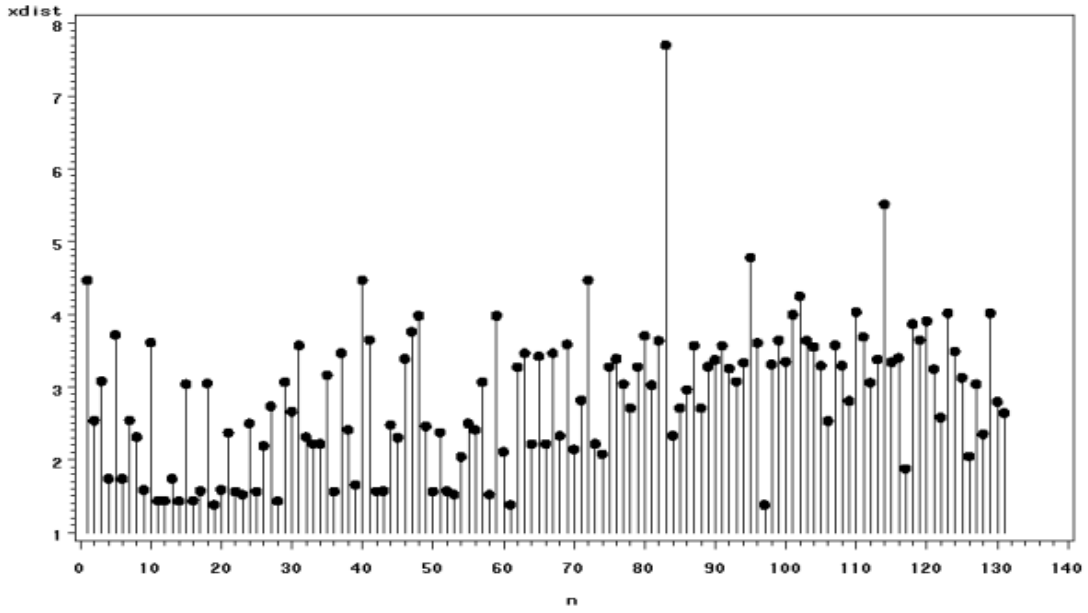

Figure 6.2 Distances from the X-variables to the initial model based on FASGAI scales.

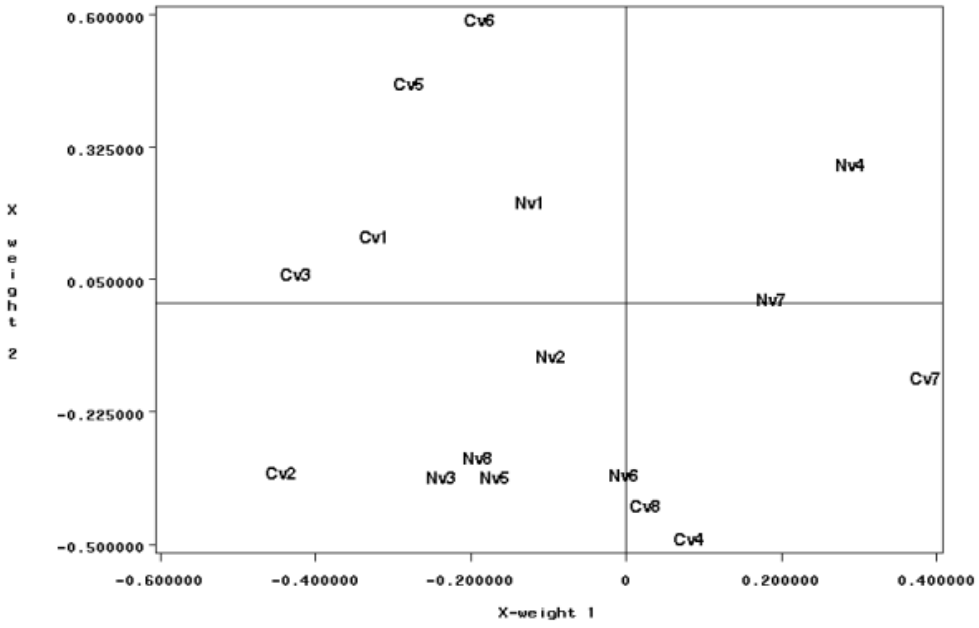

Figure 6.3 Plot of first and second X-weights for initial PLS model of ACE inhibitory dipeptides based on VHSE scales. C: C-terminial; N: N-terminal.

30 (DG), and 97(IF) were the common outliers in the four preliminary models with each model containing additional outliers. These outliers had the absolute differences between the predicted and observed activities larger than 1.5. The outliers were also examined by looking at the Euclidean distance from each point to the PLS model in both X and Y. In Figure 6.2, peptide 83 (PL) was dramatically farther from the preliminary model than the rest based on FASGAI scales (Figure 6.2). Plots of weights show which predictors are most represented in each factor and those with small weights are less important (SAS Institute Inc., Cary, NC). In Figure 6.3, the plot of X-weights for the first and second factors based on VHSE scales indicated that N-terminal v6 and C-terminal v8 (two of three vectors for electronic properties) were the least represented in factor 1 (Figure 6.3). Removing variables that were weighted at nearly zero improved the models' predictive

abilities. Variables that clustered near each other indicated their equal weight on a factor (Nash and Chaloud, 2002).

Weights were used to determine VIP (variable influence on projection) which indicated the contribution of the independent variable to the model. The regression coefficient and VIP of each variable from four sets of amino acid descriptors are concluded in Table 6.3. The predictors in Italic font could be dropped from the models since their regression coefficients (in absolute value) and VIP (<0.8) were both small (Wolt, 1995). The dropped variables tended to be the ones near zero for factor 1 in the weights plot (Figure 6.3).

The final PLS models of ACE inhibitory dipeptides

After outliers were removed, the final PLS models were derived and are showed in Table 6.4. Compared to the initial models, the root mean PRESS for each new model was significantly reduced, and the model fit and predictive ability were greatly improved. Although the extracted factors based on VHSE scales explained higher percent of the variation for the predictors, the plot of X-score and Y-score for the second factor appeared to have separate groupings (Figure 6. 4). So here the model based on FASGAI was preferred for predicting responses since the explained percent of variation for depenent variables were not significantly different. The plot of X-score and Y-score for the second factor based on FASGAI is showed in Figure 6.5. In the final model, outliers were examined by plotting the X-distance (Figure 6.6) and the Y-distance (Figure 6.7) to the model. There was no evidence of outliers in the final data. The predicted activities of ACE inhibitory dipeptides including FF isolated from Alcalase hydrolysate of cowpea are

Table 6.4 The final PLS models for ACE inhibitory dipeptides.

	Extracted factors	Root mean PRESS	Variation explained by PLS factors		Coefficient		VIP	
			independent	dependent	N	C	N	C
3-z scale	1	0.5801	39.3%	71.2%	z_1: 0.2254	0.3851	0.6703	1.1454
					z_2:	-0.4493		1.3365
					z_3: 0.2335		0.6945	
5-z scale	2	0.5971	52.8%	75.3%	z_1: 0.2949	0.3617	0.7276	1.2481
					z_2:	-0.3402		1.3834
					z_3: 0.2261		0.6572	
					z_4:	-0.0478		1.0056
					z_5:	0.1313		0.8368
FASGAI	2	0.5936	62.5%	71.9%	f1:	-0.1779		0.8722
					f2: -0.1947		0.9979	
					f3: -0.0809	-0.4459	0.6148	1.5478
					f4:	0.2387		0.9875
					f6: -0.1561		0.7827	
VHSE	2	0.5481	79.6%	76.2%	v1:	-0.1061		0.8920
					v2:	-0.2293		1.2216
					v3: -0.2231	-0.1621	0.6818	1.1687
					v4: 0.2342		0.7911	
					v5:	-0.1819		1.1654
					v7:	0.1225		1.0400

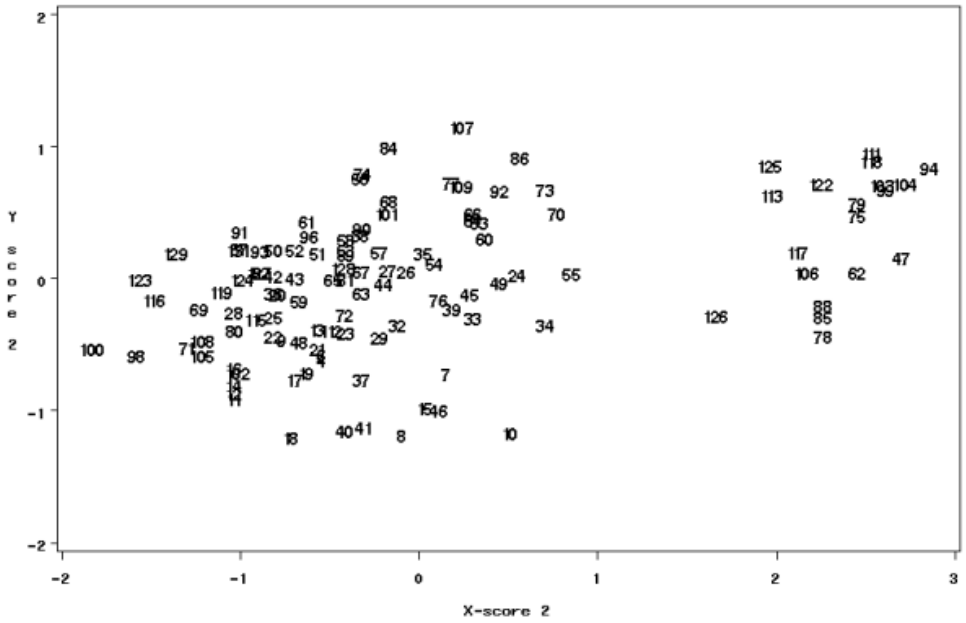

Figure 6.4 Plot of X- and Y-scores for factor 2 of the final model based on VHSE scales.

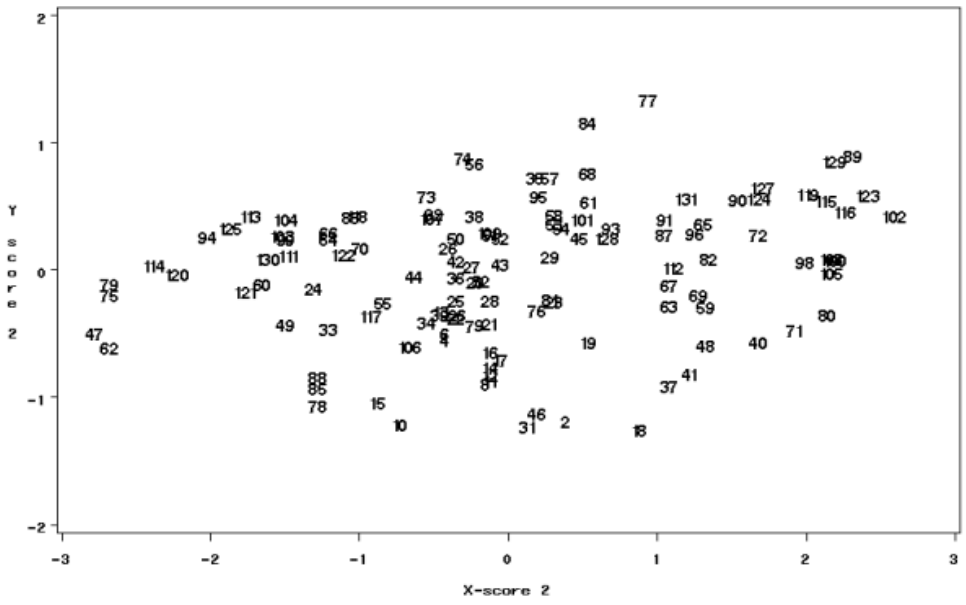

Figure 6.5 Plot of X- and Y-scores for factor 2 of the final model based on FASGAI scales.

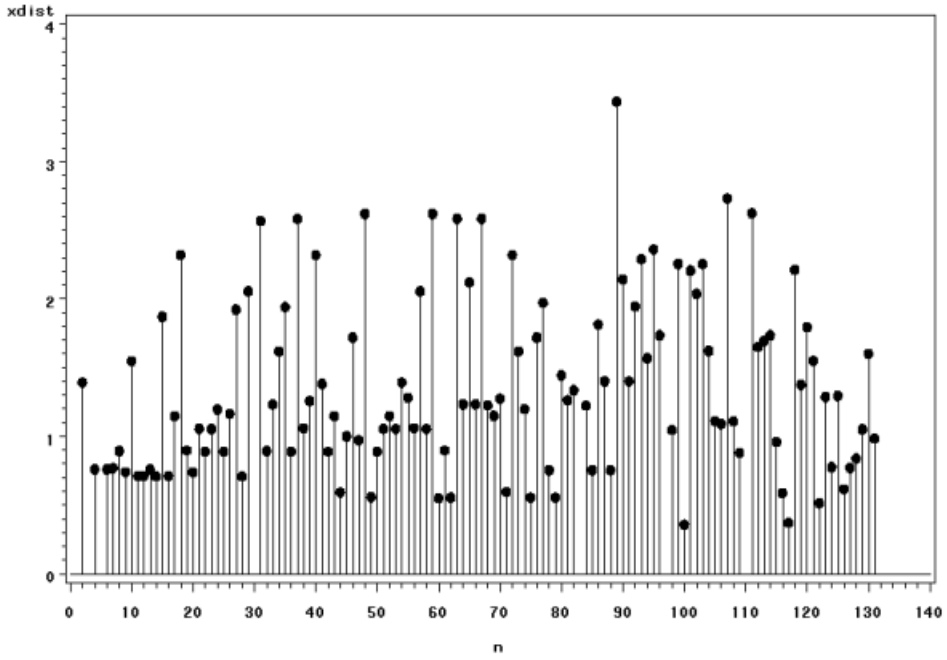

Figure 6.6 Distances from the X-variables to the final model based on FASGAI scales.

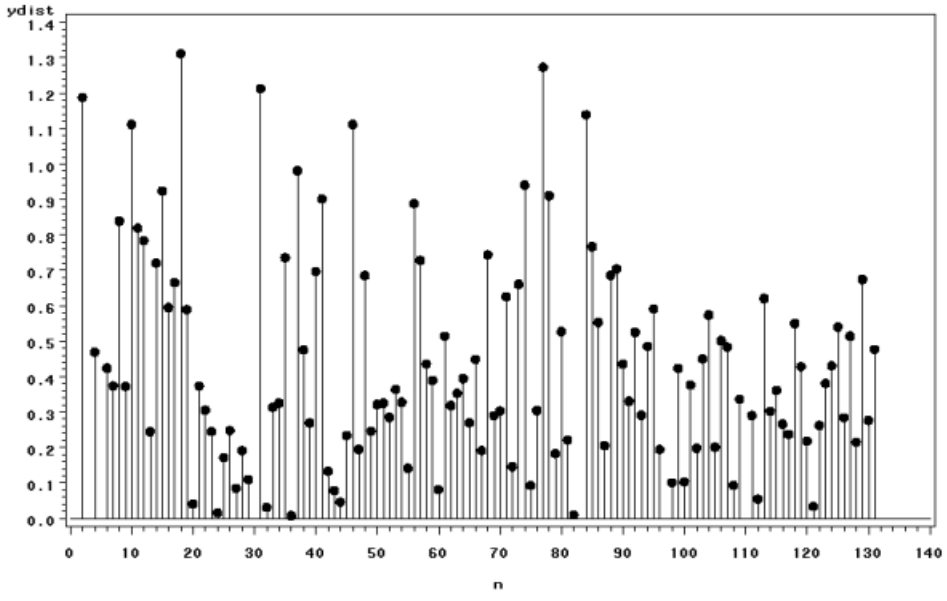

Figure 6.7 Distances from the Y-variables to the final model based on FASGAI scales.

listed in Table 6.2. The absolute differences between observed and predicted log IC_{50} are all less than 1.5. The predicted log IC_{50} for the isolated dipeptdie FF was 1.35, compared to values 1.26, 1.37 and 1.38 obtained by the models based on 3-z, 5-z and VHSE scales, respectively. They were not significantly different. The observed log IC_{50} for dipeptide FF was estimated to be 1.85. Therefore, the absolute difference between the observed and predicted responses were also smaller than 1.5.

Structure requirements of ACE inhibitory dipeptides

According to the first two models in Table 6.3 and Table 6.4 based on 3-z and 5-z scales, steric bulk property for the C-terminal amino acid residue is the most important to ACE inhibitory activity of dipeptides. Amino acids with large positive scales for steric bulk are preferred. The hydrophobicity for the C-terminal amino acid also contributes substantially to ACE inhibitory potency of dipeptides and the more hydrophobic amino acids are preferred. Referring to Appendix A and B, amino acids Trp, Phe, Tyr and Pro could simultaneously meet the two conditions for the C-terminal position, which is in agreement with the result of Cheung et al. (1980) who demonstrated that the most favorable C-terminal amino acids were Trp, Tyr, Phe and Pro. However, the z_5 property of proline can partially counteract the positive effects of steric bulk and hydrophobic properties. According to the models based on FASGAI scales, steric bulk property is still the most important. Considering two other properties f1 and f4, amino acids with more positive scales for steric bulk and hydrophobic properties and more negative scales for compositional property such as Trp, Phe and Met are preferred. The same search carried out for the models based on VHSE scales and amino acids Trp, Phe and Tyr can meet the

requirement to minimize the response log IC_{50}. In all, a large and hydrophobic amino acid at C-terminal position is conducible to ACE inhibitory activities of dipeptides. Compared to the amino acid properties at C-teminus, those at N-terminus influence ACE inhibitory potency of dipeptides to a smaller extent. The N-terminal position seems to have a preference to amino acids Val, Leu, Ile, Met and Phe according to the four models. The previous study conducted by Cheung et al. (1980) has proven that Val and Ile were the most favorable amino acids for N-termius. Interestingly, two positively charged amino acids Arg and Lys are also feasible at N-terminus according to the model based on FASGAI scales.

CONCLUSION

This research shows that a QSAR study using PLS regression can efficiently model and predict both activities and structures of ACE inhibitory dipeptides. In the context, quantitative amino acids descriptors are valuable and different scale data sets may perform in different ways. The presented results provide information for the future synthesis of active ACE inhibitory dipeptides in pharmacology and nutrition. The method can also be extended to other bioactive peptides and compounds.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

ACE is a major target for cardiovascular therapies and ACE inhibitors have been marketed for more than 20 years. However, the use of these inhibitors is hampered by common side effects. Food-derived ACE inhibitory may be more natural and safer, and could be applied in the prevention of hypertension and as initial treatment in mildly hypertensive individuals.

Peanut is an important source of arginine-rich dietary protein as well as vitamins, fiber and other nutrients. Cowpea is a starchy legume that is also an important source of dietary protein. They have showed various functional and nutritional properties. The finding of ACE inhibitory peptides can further boost the values of peanut and cowpea.

A microplate kinetic assay was established to determine ACE inhibition. ACE inhibitory potency of Alcalase hydrolysates of peanut and cowpea flours was a function of hydrolysis temperature and enzyme to substrate ratio over the ranges studied. A series of purificaions were performed and the active peptides were sequenced. Based on the dipeptides collected from previous literatures, quantitative structure-activity relationship was studied using four sets of amino acid descriptor scales, ACE inhibitory activity of a newly identified peptide was predicted and the preferred structure was proposed for ACE inhibitory dipeptides.

A number of ACE inhibitory peptidies have been isolated from various food sources though much less experiments have been conducted to investigate the *in vivo*

antihypertensive activites. The *in vitro* study is a good starting point and further research would be performed for the *in vivo* antipertentive activities of both isolated peptides from Alcalase hydrolysates of peanut and cowpea and synthesized peptides based on the proposed structure.

Appendix A Descriptor scales z_1, z_2 and z_3 for 20 coded amino acids.

Amino acid	z_1	z_2	z_3
Ala (A)	0.07	-1.73	0.09
Val (V)	-2.69	-2.53	-1.29
Leu (L)	-4.19	-1.03	-0.98
Ile (I)	-4.44	-1.68	-1.03
Pro (P)	-1.22	0.88	2.23
Phe (F)	-4.92	1.30	0.45
Trp (W)	-4.75	3.65	0.85
Met (M)	-2.49	-0.27	-0.41
Lys (K)	2.84	1.41	-3.14
Arg (R)	2.88	2.52	-3.44
His (H)	2.41	1.74	1.11
Gly (G)	2.23	-5.36	0.30
Ser (S)	1.96	-1.63	0.57
Cys (C)	0.71	-0.97	4.13
Asn (N)	3.22	1.45	0.84
ASP (D)	3.64	1.13	2.36
Thr (T)	0.92	-2.09	-1.40
Tyr (Y)	-1.39	2.32	0.01
Gln (Q)	2.18	0.53	-1.14
Glu (E)	3.08	0.39	-0.07

Appendix B Descriptor scales z_1, z_2, z_3, z_4 and z_5 for 20 coded amino acids.

Amino acid	z_1	z_2	z_3	z_4	z_5
Ala (A)	0.24	-2.32	0.60	-0.14	1.30
Arg (R)	3.52	2.50	-3.50	1.99	-0.17
Asn (N)	3.05	1.62	1.04	-1.15	1.61
Asp (D)	3.98	0.93	1.93	-2.46	0.75
Cys (C)	0.84	-1.67	3.71	0.18	-2.65
Gln (Q)	1.75	0.50	-1.44	-1.34	0.66
Glu (E)	3.11	0.26	-0.11	-3.04	-0.25
Gly (G)	2.05	-4.06	0.36	-0.82	-0.38
His (H)	2.47	1.95	0.26	3.90	0.09
Ile (I)	-3.89	-1.73	-1.71	-0.84	0.26
Leu (L)	-4.28	-1.30	-1.49	-0.72	0.84
Lys (K)	2.29	0.89	-2.49	1.49	0.31
Met (M)	-2.85	-0.22	0.47	1.94	-0.98
Phe (F)	-4.22	1.94	1.06	0.54	-0.62
Pro (P)	-1.66	0.27	1.84	0.70	2.00
Ser (S)	2.39	-1.07	1.15	-1.39	0.67
Thr (T)	0.75	-2.18	-1.12	-1.46	-0.40
Trp (W)	-4.36	3.94	0.59	3.44	-1.59
Tyr (Y)	-2.54	2.44	0.43	0.04	-1.47
Val (V)	-2.59	-2.64	-1.54	-0.85	-0.02

Appendix C Descriptor scales (VHSE) for 20 coded amino acids.

Amino acid	v1	v2	v3	v4	v5	v6	v7	v8
Ala (A)	0.15	-1.11	-1.35	-0.92	0.02	-0.91	0.36	-0.48
Arg (R)	-1.47	1.45	1.24	1.27	1.55	1.47	1.30	0.83
Asn (N)	-0.99	0.00	-0.37	0.69	-0.55	0.85	0.73	-0.80
Asp (D)	-1.15	0.67	-0.41	-0.01	-2.68	1.31	0.03	0.56
Cys (C)	0.18	-1.67	-0.46	-0.21	0.00	1.20	-1.61	-0.19
Gln (Q)	-0.96	0.12	0.18	0.16	0.09	0.42	-0.20	-0.41
Glu (E)	-1.18	0.40	0.10	0.36	-2.16	-0.17	0.91	0.02
Gly (G)	-0.20	-1.53	-2.63	2.28	-0.53	-1.18	2.01	-1.34
His (H)	-0.43	-0.25	0.37	0.19	0.51	1.28	0.93	0.65
Ile (I)	1.27	-0.14	0.30	-1.80	0.30	-1.61	-0.16	-0.13
Leu (L)	1.36	0.07	0.26	-0.80	0.22	-1.37	0.08	-0.62
Lys (K)	-1.17	0.70	0.70	0.80	1.64	0.67	1.63	0.13
Met (M)	1.01	-0.53	0.43	0.00	0.23	0.10	-0.86	-0.68
Phe (F)	1.52	0.61	0.96	-0.16	0.25	0.28	-1.33	-0.20
Pro (P)	0.22	-0.17	-0.50	0.05	-0.01	-1.34	-0.19	3.56
Ser (S)	-0.67	-0.86	-1.07	-0.41	-0.32	0.27	-0.64	0.11
Thr (T)	-0.34	-0.51	-0.55	-1.06	-0.06	-0.01	-0.79	0.39
Trp (W)	1.50	2.06	1.79	0.75	0.75	-0.13	-1.01	-0.85
Tyr (Y)	0.61	1.60	1.17	0.73	0.53	0.25	-0.96	-0.52
Val (V)	0.76	-0.92	-0.17	-1.91	0.22	-1.40	-0.24	-0.03

Appendix D Descriptor scales (FASGAI) for 20 amino acids.

Amino acid	f1	f2	f3	f4	f5	f6
Ala (A)	0.207	0.821	-1.009	1.387	0.063	-0.600
Arg (R)	-1.229	0.378	0.516	-0.328	-0.052	2.728
Asn (N)	-1.009	-0.939	-0.428	-0.397	-0.539	-0.605
Asp (D)	-1.298	-0.444	-0.584	-0.175	-0.259	-1.762
Cys (C)	0.997	0.021	-1.419	-2.080	-0.799	0.502
Gln (Q)	-0.880	0.381	-0.044	-0.455	-0.040	0.405
Glu (E)	-1.349	1.388	-0.361	0.213	0.424	-1.303
Gly (G)	-0.205	-2.219	-1.656	1.229	-1.115	-1.146
His (H)	-0.270	0.461	-0.024	-1.407	0.001	0.169
Ile (I)	1.524	0.536	0.809	0.734	-0.196	0.427
Leu (L)	1.200	1.128	0.703	1.904	0.536	-0.141
Lys (K)	-1.387	0.572	0.285	0.333	-0.169	1.157
Met (M)	0.886	1.346	0.277	-0.913	0.007	-0.265
Phe (F)	1.247	0.293	1.336	-0.026	0.012	0.015
Pro (P)	-0.407	-2.038	-0.564	0.128	3.847	-1.008
Ser (S)	-0.495	-0.847	-1.079	0.582	0.035	-0.068
Thr (T)	-0.032	-0.450	-0.610	0.341	0.117	0.577
Trp (W)	0.844	-0.075	2.069	-1.360	-0.810	-0.380
Tyr (Y)	0.329	-0.858	-1.753	-0.479	-0.835	0.289
Val (V)	1.332	0.545	0.029	1.026	-0.229	1.038