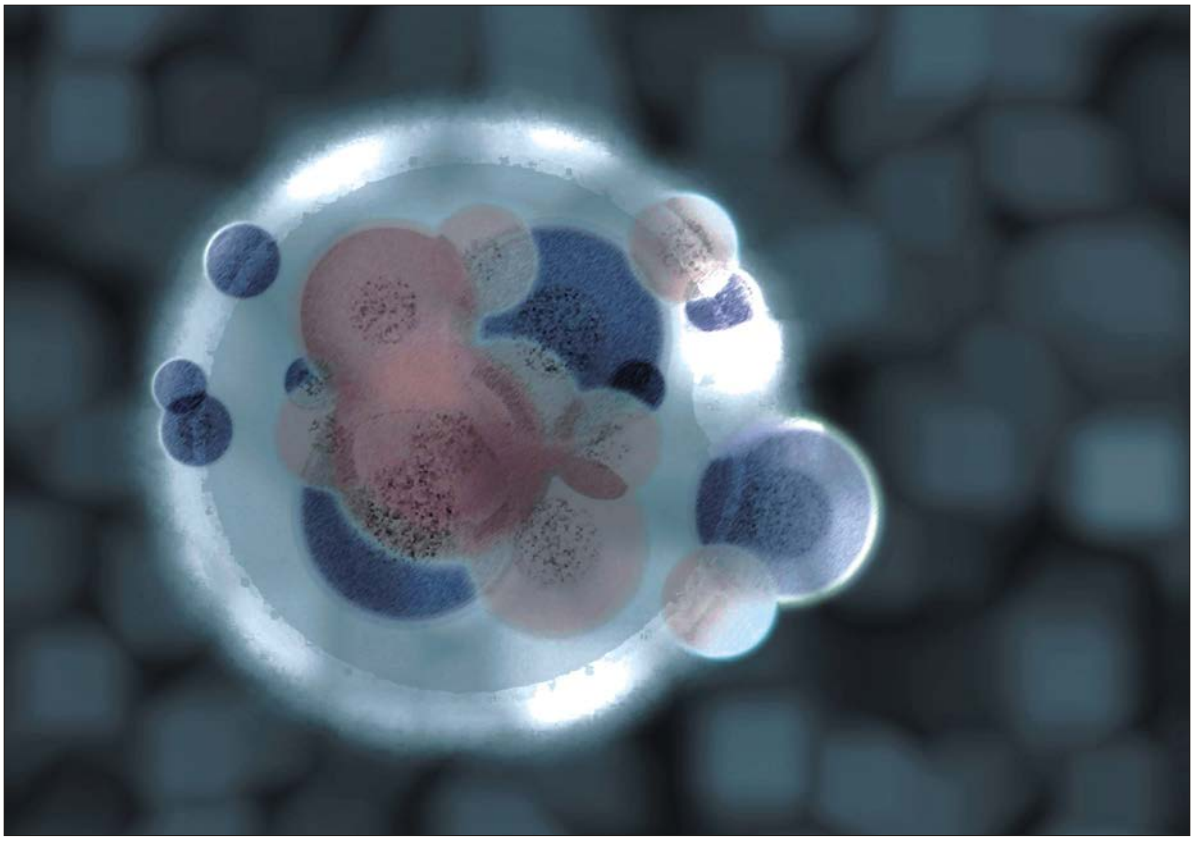

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Antihypertensive Peptides from Vicilin, the Major Storage Protein of Mung Bean (*Vigna radiata* (L.) R. Wilczek)

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Abstract: Hypertension is among the leading diseases afflicting humans and the search for cheap and alternative modes of treatment is of primary importance. This study investigated the potential of vicilin, the major storage protein of mung bean, to generate antihypertensive peptides. The total soluble proteins of mung bean var. Pag-asa 7 were extracted using 35 mM potassium phosphate buffer (pH 7.0) containing 0.40 M NaCl. Vicilin (8S globulin) was purified by a combination of ammonium sulfate fractionation, selective precipitation and gel filtration chromatography. Trypsin and chymotrypsin digests of vicilin, for a 24 h period, yielded Angiotensin Converting Enzyme (ACE) inhibitory activities of 83.95 and 93.68%, respectively. Both digests were further purified using reversed phase-high performance liquid chromatography (RP-HPLC). RP-HPLC fractions obtained from trypsin digests have IC_{50} values of 1.325, 1.151 and 1.367 mg mL^{-1} for T1, T2 and T3, respectively. Meanwhile, RP-HPLC fractions of chymotrypsin digests have IC_{50} values of 0.826, 0.203, 0.286 and 0.852 mg mL^{-1} for C1, C2, C3 and C4, respectively. Chymotrypsin was better in releasing more potent ACE inhibitory peptides than trypsin. Therefore, vicilin contains antihypertensive peptides that exhibit angiotensin converting enzyme inhibitory activities.

Key words: Mung bean, vicilin, ACE inhibitor, antihypertensive, storage protein

INTRODUCTION

Hypertension or high blood pressure, is among the leading causes of morbidity and mortality in the Philippines and Asia. In urban areas, the prevalence of hypertension may reach as high as 24% and even greater (Department of Health, 2005; Singh *et al*., 2000). The incidence of hypertension is higher in adults, however, the disease has also been observed to inflict lower age groups (Reyes-Gibby and Aday, 2000; Micozzi, 1980).

One aspect of regulating blood pressure is through the renin-angiotensin system involving the angiotensin I converting enzyme (ACE). ACE converts the inactive decapeptide angiotensin I to angiotensin II, a potent vasoconstrictor, as well as inactivating the vasodilator bradykinin. Hence, inhibition of the ACE activity will ultimately result to a lowering of blood pressure (Natesh *et al*., 2003; Unger, 2002).

In recent years, numerous bioactive peptides with antihypertensive effects have been isolated from dietary proteins. These peptides are often contained in the parent protein and can only exert their biological activities upon release. Among the food sources of antihypertensive peptides include those from plants such as soybean (De Mejia and De Lumen, 2006) and animals such as milk (Jakala and Vapaatalo, 2010) and egg (Miguel and Aleixandre, 2006). Hence, food sources of bioactive peptides are of utmost significance since these are natural, cheap and readily available materials.

Mung bean (*Vigna radiata* (L.) R. Wilczek) is an excellent source of food protein with a protein content amounting to 25% (Butt and Batool, 2010). The major storage proteins of mung bean are the globulin (62%), albumin (16.3%), glutelin (13.3%) and prolamin (0.9%) (Tsou *et al*., 1979). The vicilin type protein (8S) accounts for 89% of the total globulins and contains disulfide linkages as well as carbohydrates (Mendoza *et al*., 2001).

The focus of this study was to determine whether vicilin contains antihypertensive bioactive peptides that can be released by enzymatic digestion using trypsin and chymotrypsin.

MATERIALS AND METHODS

Sample preparation: Mung bean seeds of variety Pag-asa 7 were obtained from the National Seed Foundation,

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The coats of mung bean seeds were manually removed with scalpel blade. The dehulled seeds were ground into flour using a mortar and pestle and defatted with *n*-hexane (1 g ground seeds: 10 mL hexane) for one hour in an ice bath with constant stirring. The resulting mixture was allowed to stand for 3 min. The solvent was removed by decantation, while the residue was air-dried and stored at 4°C until use.

Extraction of proteins: The total soluble proteins of mung bean were extracted according to the method of Kortt (1986), with some modifications. Fourteen grams of defatted mung bean flour was added with 280 mL of 35 mM potassium phosphate buffer (pH 7.0) containing 0.40 M NaCl. The mixture was stirred for 1 h in an ice bath then clarified by centrifugation at 12000 rpm for 5 min at 4°C using an Eppendorf 5415C Benchtop centrifuge. The supernatant or clarified crude extract was collected and stored at 4°C until use.

Purification of vicilin

Ammonium sulfate fractionation: Cooper's nomogram (Cooper, 1977) was used in the fractionation of the crude extract. Initially, 18.15 g solid ammonium sulfate was added to 75 mL of crude extract to achieve 40% saturation. The mixture was gently stirred for 30 min on ice and then centrifuged at 10000 rpm for 10 min at 4°C. The precipitate was collected and the supernatant was further added with 9.75 g of ammonium sulfate to achieve 60% saturation. The mixture was again stirred for 30 min on ice and allowed to stand overnight at 4°C. The supernatant was recovered by centrifugation at 10000 rpm for 10 min at 4°C.

Selective precipitation: The supernatant at 60% ammonium sulfate saturation was dialyzed for 48 h against distilled water containing 10 mM β-mercaptoethanol. The dialysate was centrifuged at 12000 rpm for 5 min at 4°C to collect the globulin precipitate. The precipitate was resuspended in minimum amount of extraction buffer and then subjected to gel filtration chromatography.

Gel filtration chromatography: A 0.60 mL globulin fraction with a protein content of 3.23 mg mL^{-1} was loaded onto a Sephadex G-150 column (1×30 cm) (Sigma-Aldrich). The sample was eluted with the extraction buffer at a flow rate of 0.33 mL min^{-1}. Eluted proteins were monitored by measuring the absorbance at 280 nm using a Bio-Rad SmartSpec™ 3000 spectrophotometer. Fractions (1 mL) were routinely analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions with the same band in SDS-PAGE were pooled and concentrated by freeze drying.

Enzymatic protein digestion: The vicilin fraction was subjected to enzymatic hydrolysis using trypsin and chymotrypsin. A 200 µL enzyme solution (1 µg mL^{-1}) was added to 200 µL of the protein sample (0.841 mg mL^{-1}). Digestion was allowed to proceed for 24 h at 25°C. The enzyme was inactivated by heating the mixture at 100°C for 5 min. After heating, the mixture was immediately transferred and kept in ice. The efficiency of the enzymatic digestion was determined through SDS-PAGE.

Reverse-phase high performance liquid chromatography (RP-HPLC): Fractionation of the tryptic and chymotryptic digests was done according to the method of Silva *et al.* (2006). RP-HPLC was performed on Agilent technologies 1200 Series HPLC system, using a Vydac C18 column (4.6×250 mm, 5 µm bead, 30 nm pore size) with a guard column and disposable cartridge (10 nm, 12 µm).

A 20 µL sample was injected into the column and eluted at 40°C via a mobile phase of 2 solvents-solvent A: 0.05% (v/v) Trifluoroacetic Acid (TFA) in water; and solvent B: 0.05% (v/v) TFA in acetonitrile:water (9:1). Elution was started with 98% (v/v) solvent A, a linear gradient from 2 to 60% (v/v) solvent B over 45 min, another linear gradient from 60 to 100% (v/v) solvent B for 2 min, 100% (v/v) solvent B for 5 min, a linear gradient from 98 to 2% (v/v) solvent B for 2 min, then ending with 2% (v/v) solvent B for 2 min. The flow rate was set at 0.5 mL min^{-1}. Detection was done using a UV detector at 215 nm. The fractions were collected based on their peak assignments. All samples were subjected to microfiltration prior to injection.

Characterization of proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): The protein samples were subjected to SDS-PAGE on a Bio-Rad mini gel electrophoresis apparatus according to the method of Laemmli (1970). The run was carried out in 11% denaturing gel at 90 V for 2 h. The molecular weights of the subunits were estimated using Trail Mix™ Protein Markers. The gel was stained using 0.05% Coomassie Brilliant Blue R-250 for 30 min and then destained using an aqueous solution of methanol and acetic acid until the bands were distinct.

Determination of protein content: The protein content of the samples was determined using the Bradford (1976) method with Bovine Serum Albumin (BSA) as protein standard.

Extraction of ACE from rabbit lungs: Extraction of ACE from rabbit lungs was done according to the method of Cushman and Cheung (1971) with minor modifications. Rabbit lungs were ground using liquid nitrogen and then homogenized in acetone (1 g lung: 2 mL acetone) using a blender. The homogenate was centrifuged at 7000 rpm for 30 min using Sorvall® RC 5B PLUS Centrifuge at 4°C and then allowed to stand until the acetone has evaporated. The residue was dissolved in 100 mM phosphate buffer, pH 8.3 (1 g residue: 10 mL buffer). The resulting solution was centrifuged at 4000 rpm and 4°C for 75 min. The clear supernatant was then collected and stored at -20°C until use.

Spectrophotometric assay for ACE activity: Determination of the ACE inhibitory activities of the digests was done according to the method of Cushman and Cheung (1971) with minor modifications. Fifty μL of the protein sample was added to 250 μL mixture containing 50 μL of 100 mM phosphate buffer (pH 8.3), 50 μL of 300 mM NaCl, 100 μL of 5 mM Hippuryl-L-Histidyl-L-Leucine (HHL) and 50 μL of ACE. The mixture was incubated at 37°C for 30 min using a New Brunswick Scientific G24 Environmental Incubator Shaker. The reaction was stopped by adding 250 μL of 1 N HCl. The reaction mixture was then added with 1.5 mL of ethyl acetate and mixed through vortex for 15 s. A 1.0 mL aliquot of the ethyl acetate layer was obtained then the solvent was allowed to evaporate. The residue was redissolved in 1.0 mL of distilled water and the absorbance of the resulting solution at 228 nm was determined. For the blank, no peptide sample was added. The negative control was devoid of peptide sample and HCl was added prior to ACE. Pulverized captopril served as positive control.

ACE inhibitory activities were expressed as inhibitory activity (U), percent inhibition and IC_{50} values. All values are means of three trials.

RESULTS AND DISCUSSION

Extraction and purification of vicilin from mung bean: Vicilin, an 8S type globulin, was purified by a combination of ammonium sulfate fractionation, selective precipitation and gel filtration chromatography.

The optimum ammonium sulfate saturation which removed most of the protein contaminants was found to be 60% (Fig. 1). Vicilin was present in the supernatant and further removal of minor storage proteins was achieved during dialysis where globulins were selectively precipitated while albumins remain solubilized. When the precipitated globulin fraction was subjected to gel filtration chromatography, only one major peak was observed, corresponding to the purified vicilin fraction (Fig. 2). Mung bean vicilin consists of four subunits with molecular weights of 66.3, 50.0, 29.5 and 24.0 kDa on SDS-PAGE. This profile is also similar to the vicilin fraction obtained by Ericson and Chrispeels (1973) for that of *Phaseolus aureus*, with molecular weights of 63.5, 50.0, 29.5 and 24.0 kDa. However, mung bean vicilin lacks lower molecular weight subunits like those found in pea (Gatehouse *et al*., 1981), Coconut (Garcia *et al*., 2005) and Brazil Nut (Sharma *et al*., 2010). Mung bean contains

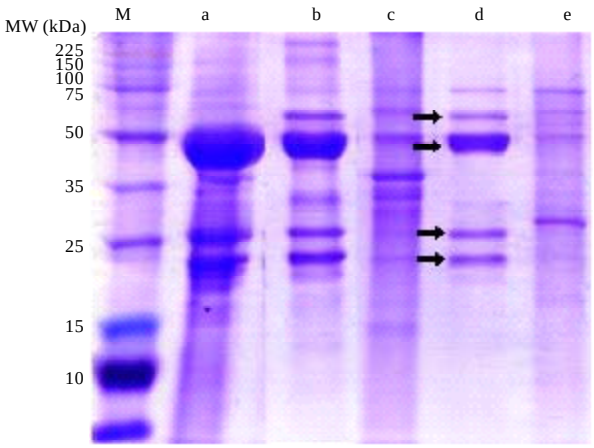

Fig. 1: SDS-PAGE profiles of fractions obtained from ammonium sulfate precipitation, Lane M: Molecular weight markers, Lane a: Total crude extract, Lane b: 40% ammonium sulfate supernate, Lane c: 40% ammonium sulfate precipitate, Lane d: 60% ammonium sulfate supernate, Lane e: 60% ammonium sulfate precipitate, The vicilin subunits are indicated by arrows

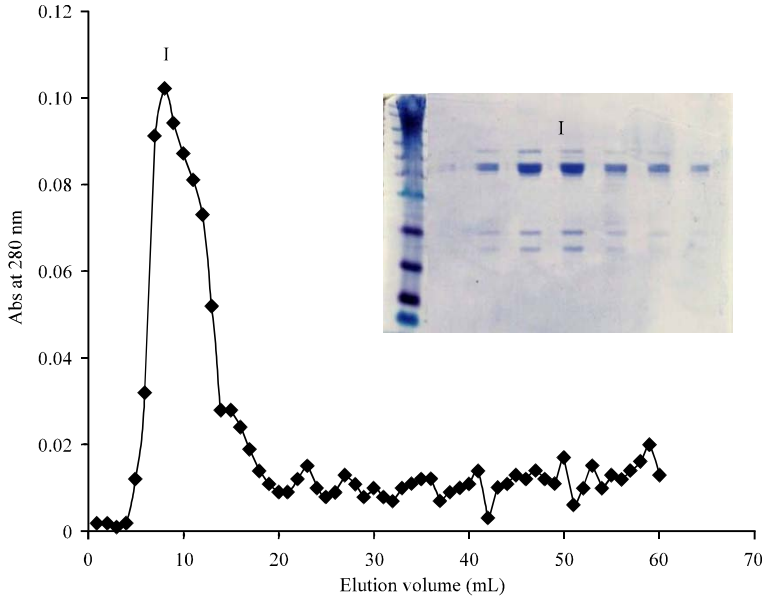

Fig. 2: Gel filtration chromatography profile of mung bean vicilin on Sephadex G-150 column. Peak I: Vicilin, 8S type globulin; Inset-SDS-PAGE profile of gel filtration chromatography fractions from peak I

various types of storage proteins such as albumins and globulins. Mendoza *et al*., (2001) earlier reported that vicilin comprise majority of the mung bean proteins amounting to 89% of the total globulins, while the 11S and basic 7S comprise only 7.6% and 3.4, respectively.

Enzymatic digestion of vicilin and preliminary ACE inhibition assay: The purified vicilin fraction was subjected to enzymatic hydrolysis for 24 h using trypsin and chymotrypsin. Digestion was fairly complete for both enzymes after 24 h reaction as shown by the absence of protein bands on SDS-PAGE (Fig. 3). This implies that vicilin has been converted to very small molecular weight peptides that are not resolved and retained by SDS-PAGE. Guang and Phillips (2009) reported that small molecular weight peptides with short sequences have the greatest potential to exhibit ACE inhibitory activities since they fit easily into the active site of the angiotensin-converting enzyme. Wang and Mejia (2005) observed that many of these bioactive peptides are of low molecular weight and typically consist of amino acids ranging from 2 to 20 or more. Furthermore, the use of long hydrolysis times, such as a 24 h period, generally results to the production of ACE inhibitory peptides with more potent activities (Hyun and Shin, 2000).

Preliminary ACE inhibition assay shows that both trypsin and chymotrypsin digests yielded peptides that inhibit ACE activity (Table 1). The highest ACE inhibition activity was observed for that of the chymotryptic digests

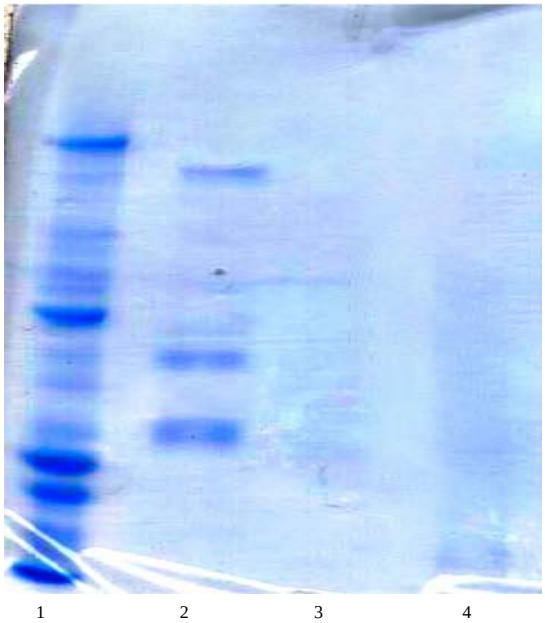

Fig. 3: Enzymatic digestion of vicilin for 24 h period using chymotrypsin and trypsin, Lane 1: Molecular weight markers, Lane 2: Purified vicilin fraction, Lane 3: Trypsin-treated vicilin, Lane 4: chymotrypsin-treated vicilin

with 93.68% followed by tryptic digests with 83.95%. The relatively high inhibitory activities of the protein hydrolysates may be attributed to the generation of

various kinds of bioactive peptides exerting synergistic effects. It has also been observed that using a single enzyme during the hydrolysis of the parent protein yielded peptides with high ACE inhibitory activities (Chiang *et al*., 2008; Li *et al*., 2007), even higher than those produced by multiple proteases (Yang *et al*., 2004; Yokohama *et al*., 1992). On the other hand, Wei and Chiang (2009) showed that more potent bioactive peptides were generated from porcine blood proteins with the use of a combination of proteases.

These results also demonstrate the presence of ACE inhibitory peptides encrypted in the vicilin protein. Like many other plant-derived bioactive peptides such as those from rice, corn, soybean and wheat (Guang and Phillips, 2009; Hong *et al*., 2008), mung bean storage proteins are good sources of antihypertensive peptides that may lead to the prevention and treatment of hypertension.

Table 1: ACE inhibitory activities of the chymotrypsin-and trypsin-treated vicilin

Sample	Remaining ACE activity (IU), $\times10^{-3}$	Inhibitory activity (IU), $\times10^{-3}$	Inhibition (%)
Tryptic digest	0.342±0.070	1.786±0.070	83.95±3.30
Chymotryptic digest	0.134±0.057	1.994±0.057	93.68±2.67

Unit activity of ACE: 2.128×10^{-3} IU, 1 IU: Amount of ACE (μmol) catalyzing the formation of 1 μmol of hippuric acid from HHL in 1 min at 37°C, under standard assay conditions

Purification of bioactive peptides through RP-HPLC: Peptides generated from enzymatic hydrolysis were further subjected to RP-HPLC separation. Figure 4 shows the RP-HPLC profiles of the tryptic and chymotryptic digests as well as the fractions exhibiting ACE inhibitory activities (Table 2). It should be noted that while some peptides from the chymotryptic digests have similar retention times with that of the tryptic digests indicating comparable polarities, the differences in inhibitory activities of these peptide fractions may lie on the differences in the amino acid sequence. The most potent inhibitory activities were observed for both chymotrypsin digests C2 and C3, with IC_{50} values of 0.203 and 0.286 mg mL^{-1}, respectively. On the other hand, the

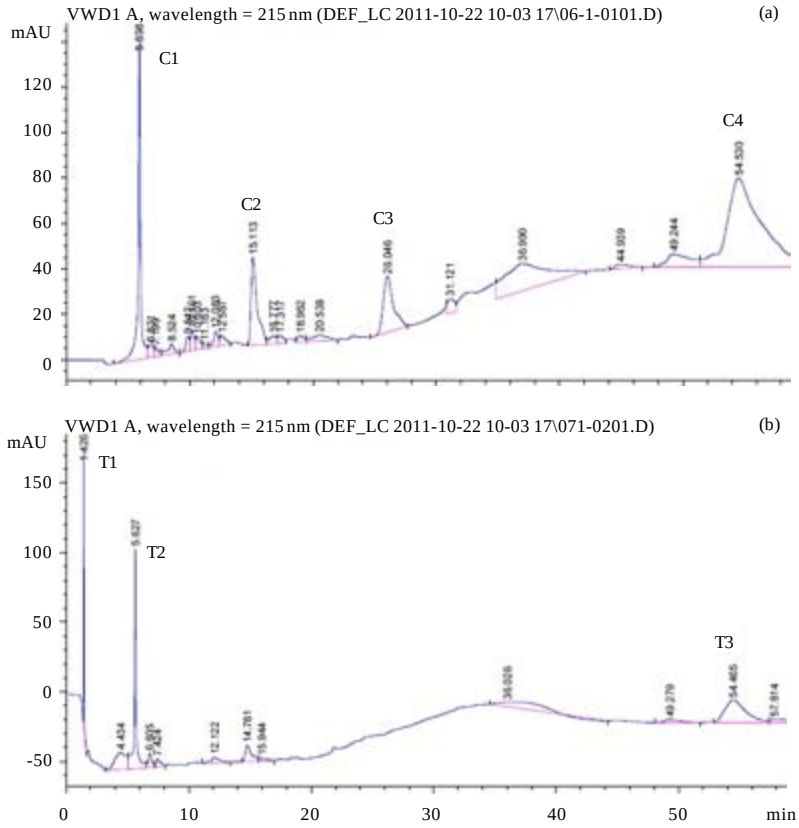

Fig. 4(a-b): RP-HPLC profiles of chymotryptic and tryptic digests on Vydac C18 column (a) Chymotryptic digests showing fractions C1, C2, C3 and C4 and (b) Tryptic digests showing fractions T1, T2 and T3

Table 2: ACE inhibitory activities of fractions obtained from RP-HPLC

Fraction	Enzyme	Inhibition (%)	IC_{50}
C1	Chymotrypsin	49.03±0.36	0.826±0.046
C2	Chymotrypsin	78.87±0.49	0.203±0.014
C3	Chymotrypsin	74.05±1.15	0.286±0.007
C4	Chymotrypsin	40.35±1.55	0.852±0.027
T1	Trypsin	25.49±0.71	1.325±0.142
T2	Trypsin	33.88±0.49	1.151±0.005
T3	Trypsin	7.15±0.36	1.367±0.016
(+)-control	Captopril	96.75±0.80	0.028±0.001

Values are mean of three trials, Unit of IC_{50} is in μM for captopril and mg mL^{-1} for protein hydrolysates

tryptic digests yielded the least inhibitory activities with IC_{50} values of 1.325 and 1.367 mg mL^{-1} for T1 and T3, respectively. The results also suggest that chymotrypsin is more effective in releasing ACE inhibitory peptides than trypsin. Furthermore, it can also be seen that the ACE inhibitory activities of the tryptic and chymotryptic digests were less potent than that of the commercial antihypertensive peptide captopril. The C2 fraction which exhibited the highest inhibitory activity was about 20% less potent than captopril.

The consumption of mung bean satisfies not only the protein requirements of an individual but also offers advantages in the well-being and prevention of diseases. Hypertension may be alleviated through the enzymatic hydrolysis of vicilin in the small intestines upon ingestion of mung bean.

CONCLUSION

Vicilin, the major storage protein of mung bean, was purified by a combination of ammonium sulfate fractionation, selective precipitation and gel filtration chromatography. Vicilin contains bioactive peptides that inhibit ACE activity and may therefore exhibit antihypertensive effects. These peptides can be released from the parent protein by enzymatic hydrolysis using trypsin and chymotrypsin. The RP-HPLC fractions of chymotrypsin digests yielded peptides with more potent ACE inhibitory activities than that of the trypsin digests. Further study should focus on the determination of the sequence of these peptides as well as their antihypertensive effects *in vivo*.

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