JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Angiotensin I-Converting Enzyme Inhibitory Peptide Derived from Glycinin, the 11S Globulin of Soybean (*Glycine max*)

K. G. Mallikarjun Gouda, Lalitha R. Gowda, A. G. Appu Rao,* and V. Prakash

Department of Protein Chemistry and Technology, Central Food Technological Research Institute, MYSORE 570 020, India

Angiotensin I-converting enzyme (ACE), a dipeptidyl carboxypeptidase, catalyzes the conversion of Angiotensin I to the potent vasoconstrictor Angiotensin II and plays an important physiological role in regulating blood pressure. Inhibitors of angiotensin 1-converting enzyme derived from food proteins are utilized for pharmaceuticals and physiologically functional foods. ACE inhibitory properties of different enzymatic hydrolysates of glycinin, the major storage protein of soybean, have been demonstrated. The IC_{50} value for the different enzyme digests ranges from 4.5 to 35 μg of N_2. The Protease P hydrolysate contained the most potent suite of ACE inhibitory peptides. The ACE inhibitory activity of the Protease P hydrolysate after fractionation by RP-HPLC and ion-pair chromatography was ascribed to a single peptide. The peptide was homogeneous as evidenced by MALDI-TOF and identified to be a pentapeptide. The sequence was Val-Leu-Ile-Val-Pro. This peptide was synthesized using solid-phase FMOC chemistry. The IC_{50} for ACE inhibition was 1.69 ± 0.17 μM. The synthetic peptide was a potent competitive inhibitor of ACE with a K_i of $4.5 \pm 0.25 \times 10^{-6}$ M. This peptide was resistant to digestion by proteases of the gastrointestinal tract. The antihypertensive property of this peptide derived from glycinin might find importance in the development of therapeutic functional foods.

KEYWORDS: Angiotensin-converting enzyme; angiotensin-converting enzyme inhibitor; soybean; *Glycine max*; glycinin; peptide

INTRODUCTION

Hypertension is a growing undesired symptom that damages health and threatens mostly the developed societies. It is one among the major independent risk factors for atherosclerosis, stroke, myocardial infarction, and end stage renal disease. Angiotensin I-converting enzyme (EC 3.4.15.1 ACE), a dipeptidyl-dipeptidase, is the key enzyme that functions in the rennin-angiotensin system to increases blood pressure (*1*). ACE increases blood pressure by both converting the inactive decapeptide Angiotensin I to the potent vasoconstrictor Angiotensin II and inactivating the vasodilator bradykinin. Inhibition of ACE is considered the first line of therapy for hypertension and atherosclerosis. Since the original discovery of ACE inhibitors in snake venom (*2*), pharmacological orally active ACE inhibitors captopril (D-3-mercapto-2-methylpropionyl]-L-proline), enalapril, and lisinopril have been developed and are currently in use. These inhibitors have short peptides that bind tightly to the active site of ACE competing with Angiotensin I for occupancy (*3*, *4*). Captopril is an alanyl proline derivative containing a sulfhydryl group that increases its affinity for the ACE active site. These synthetic inhibitors of ACE have established themselves in the therapy of hypertension and congestive heart failure (*5*). Synthetic ACE inhibitors are remarkably effective, but they cause adverse side effects, and therefore, the trend has been toward developing safer, natural ACE inhibitors.

Nutraceutical function of foods for use in therapeutic formulations is drawing considerable attention (*6*). Interest has recently been directed to the identification, isolation, and purification of ACE inhibitory peptides from enzymatic hydrolysates of food proteins. Enzymatic protein hydrolysates containing short-chain peptides with characteristic amino acid composition and defined molecular size are desirable for specific formulations and end use (*7*). ACE inhibitory peptides derived from daily dietary food proteins would be useful in the development of a novel functional food additive and present a healthier and natural alternative to ACE inhibitor drugs. ACE inhibitory activity of peptides in vitro or in vivo has been demonstrated in casein (*8*), fish muscle (*9*), zein (*10*), soy sauce (*11*), fermented milk (*12*), mushroom (*13*), and rapeseed (*14*). The beneficial effects of soybean (*Glycine max*) protein in the prevention of hyperlidemic or hypercholesterolomic lesions that cause arteriosclerosis and hypertension are reported (*15*). Peptides from fermented soybean paste (*16*) and soybean hydrolysate (*17*) exerted an inhibitory activity of ACE in vascular tissue in vivo and lowered systolic blood pressure. Four ACE inhibitory peptides isolated from the peptic digests of

* Corresponding author. Tel.: +91-821-2515331; fax: +91-821-2517233; e-mail: appu@cftri.res.in.

10.1021/jf060264q CCC: $33.50
Published on Web 06/02/2006

soybean protein showed relatively high IC_{50} values to the order of 15 mM (*18*, *19*). In comparison to milk proteins, the research on ACE inhibitory peptides from soybean is incomplete. Considering the diversity and complexity of the different protein types of soybean, soy protein provides an abundant source of biologically active peptides. Many potential antihypertensive peptide sequences are embedded in the available protein sequence data for glycinin, the major storage protein and 11S globulin of soybean. This potential is yet to be realized. There exists therefore a great potential for releasing these bioactive peptides from their latency using proteases of varied specificity. In the present investigation, proteolysis of glycinin was engineered using varied proteases to harness peptides that exhibit ACE inhibitory activity. Accordingly, the Protease P hydrolysate contained a suite of very potent ACE inhibitory peptides that were subsequently fractionated and purified. A significant fraction of the ACE inhibitory activity could be ascribed to a pentapeptide, Val-Leu-Ile-Val-Pro (VLIVP).

MATERIALS AND METHODS

Materials and Chemicals. Soybean (*G. max*) was obtained from the local market. Bovine pancreatic trypsin (2× crystallized, type III EC 3.4.21.4, 1400 units/mg), bovine pancreatic α-chymotrypsin (3× crystallized, type II EC 3.4.21.1, 51 units/mg), pepsin A from porcine stomach mucosa (EC 3.4.23.1, 2730 units/mg of protein), pancreatin from porcine pancreas, trifluoroacetic acid (TFA), hippuryl-histidyl-leucine (HHL), hippuric acid, 1,2-ethanedithiol, and pentafluoropropionic acid were obtained from Sigma Chemical Co., St. Louis, MO. Protease-P-Amano-P was from Amano Pharmaceuticals, Nagoyo, Japan. Ginger protease was prepared from an acetone powder of ginger (*Zingiber officinale*) as described earlier (*20*). Amino acid standards and phenylisothiocynate (PITC) were from Pierce Chemical Company, Rockford, IL.

9-Fluorenylmethyloxycarbonyl (FMOC)-protected L-amino acids, 4-(2′,4′-dimethoxyphenyl-FMOC-aminomethyl)phenoxyacetamido MBHA resin, 100−200 mesh, substitution 0.45 meq/g (Rink amide MBHA), and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were obtained from CS Bio Co, San Carlos, CA. All other reagents and solvents used were of analytical/HPLC grade.

Isolation of Glycinin. Glycinin was isolated as described earlier (*21*). A 10% (w/v) slurry of defatted soybean flour in water containing β-mercaptoethanol (0.1% v/v) was extracted by agitation for 4−6 h at ambient temperature and clarified by centrifugation at 6000−8000 rpm for 45 min at 25 ± 2 °C. Glycinin was precipitated by the addition of solid $MgCl_2$ to a final concentration of 5 mM. The precipitated glycinin was collected by centrifugation and freeze-dried. Glycinin was stored at 4 °C until further use.

Preparation and Purification of Glycinin Derived ACE Inhibitor Peptides. One gram of isolated glycinin was suspended in 5 mL of 0.1 M Tris-HCl buffer, pH 8.2, and then digested with Protease P (Amano-P from *Aspergillus* sp (2200 units/mg of protein)) using an enzyme−substrate ratio of 2% (w/w) at 37 °C for 18 h. The pH of the digestion mixture was maintained at pH 8.2 by the addition of 1 M NaOH. The reaction was terminated by the addition of 100% (w/w) trichloroacetic acid (TCA) to a final concentration of 10% (w/w) and cooled on ice. The hydolysate was centrifuged at 15 000 rpm for 30 min at 4 °C. This suite of peptides was used as the source of ACE inhibitory peptides. The supernatant containing a suite of peptides was further fractionated by RP-HPLC on a C-18 Shimpak column (250 × 4.6 mm, 5 μM) using a binary gradient of 0.1% TFA and 70% acetonitrile in water containing 0.05% TFA at a flow rate of 0.7 mL/min traversing from 0 to 35%. The peptides were detected at 230 nm. ACE inhibitor peptides were also prepared by digesting glycinin with either bovine trypsin or chymotrypsin at an enzyme−substrate ratio of 2% (w/w) at 37 °C for 18 h using the same buffer as described previously. However, for ginger protease (290 units), the digestion buffer used was 0.1 M sodium phosphate pH 6.0 containing 0.6 mM DTT and was incubated at 50 °C for 16 h.

Purification and Characterization of the ACE Inhibitor Peptides. The most potent fraction was further purified on the same reverse-phase column using a gradient of (A) 50 mM ammonium acetate and (B) 50 mM ammonium acetate/acetonitrile (50:50). The peptide fraction showing highest ACE inhibition was subjected to RP-HPLC on a Shimpak C-18 column using TFA/acetonitrile solvent as described previously. The homogeneity of the peptide 3−2 was ascertained by Edman degradation using an Applied Biosystems 470 automated gas-phase sequencer equipped with an online detection system for PTH-amino acids. The release of a single amino-terminal amino acid indicates its homogeneity. The homogeneity and size of the peptide was also evaluated by MALDI-TOF.

In Vitro Assay of ACE Inhibitory Activity. The ACE inhibitory activity was assayed by RP-HPLC modified from the spectrophotometric method (*22*) as reported by Wu and Ding (*23*). ACE was extracted from porcine lung and kidney acetone powder prepared in the laboratory. One gram of acetone powder was extracted with 10 mL of sodium borate buffer, pH 8.3, containing 300 mM NaCl at 4 °C for 16−18 h. The extract was centrifuged at 15 000*g* for 60 min at 4 °C. The supernatant was dialyzed against the same buffer for 24 h (500 mL × 3). The specific activities of the porcine and lung ACE were 0.11 and 0.15 units/mg of protein. ACE activity was assayed by monitoring the release of hippuric acid from the substrate HHL at 228 nm. The assay mixture contained the following in a total volume of 0.45 mL: 0.1 mL of 0.1 M borate buffer, pH 8.3, containing 1% (w/w) NaCl; 0.05 mL of 5 mM HHL; and 0.025 mL of ACE enzyme extract. The reaction was arrested after incubation at 37 °C for 30 min by the addition of 0.25 mL of 1 N HCl. The product hippuric acid was separated from HHL by a RP-HPLC Shimpak octadecyl column (250 × 4.6 mm, 5 μM) by isocratic elution with 50% methanol containing 0.1% TFA at a flow rate of 0.8 mL/min and detected at 228 nm. One unit of ACE is defined as that amount of enzyme, which releases 1 μmol of hippuric acid per hour at 37 °C and pH 8.2. To determine the inhibition, the enzyme was preincubated with the peptide fractions. The IC_{50} value is defined as the concentration of peptides required to decrease the ACE activity by 50%. The concentration of the peptide mixture was expressed as micrograms of N_2, determined by the copper catalyst Kjeldhal method (*24*). The percent inhibition curves were plotted using a minimum of three determinations for each concentration and IC_{50} values derived from the semilogarithmic plots. A linear regression analysis was performed with the software Origin 4.1.

Inhibitory Kinetics. Various concentrations of the substrate HHL were incubated with either porcine or kidney ACE in the presence/absence of the purified peptide at 37 °C. The hippuric acid released was determined as described previously. A calibration curve for standard hippuric acid was constructed for reference. The initial reaction velocities were calculated from the hippuric acid released. The type of inhibition was determined from a Lineweaver−Burk plot (*25*).

Peptide Synthesis. The amide form of the pentapeptide corresponding to the isolated ACE inhibitory peptide was synthesized. Synthesis of the peptide was carried out in a 0.45 mmol scale by the solid-phase procedure using FMOC-protected amino acids. The peptide was assembled using Rink amide MBHA resin (0.45 meq/g) as solid support, on an automatic peptide synthesizer Model CS136 from CS Bio Co (San Carlos, CA). The FMOC-protecting group was removed with 20% (v/v) piperidine in dimethyl formamide (DMF). The peptide-coupling agent HBTU was used to activate the incoming carboxy group and to suppress racemization. Before cleavage, FMOC was removed from the terminus, and the resin was washed thoroughly with DMF.

The pentapeptide was obtained after cleavage of the peptidyl-resin with TFA/ethanedithiol/water 5:0.125:0.125 (v/v) for 2 h at room temperature. The resin was filtered into 100 mL of cold diethyl ether. The precipitated crude peptide was collected and washed 3 times with diethyl ether. The residual ether was removed by evaporation under reduced pressure. The peptide was purified by RP-HPLC on a C-18 Symmetry Shield column (Waters Associate) gradient eluted with 0.1% TFA/CH_3CN at 0.7 mL/min.

Amino Acid Analysis. The isolated and synthesized peptides were hydrolyzed in vacuo at 110 °C in 5.8 M HCl for 24 h. Amino acid analysis was performed by precolumn derivatization using PITC. The

Table 1. IC_{50} Values for ACE Inhibition of Glycinin Hydrolysates

protease	IC 50 (μg N_2) porcine lung ACE	porcine kidney ACE
bovine trypsin	9.0 ± 0.5	18.4 ± 0.6
bovine chymotrypsin	29.0 ± 1.8	27.2 ± 1.0
ginger protease	35.1 ± 1.5	33.6 ± 1.7
Protease P	4.5 ± 0.2	4.9 ± 0.4

phenyl thiocarbamoyl amino acids were analyzed by HPLC using a Waters Associate Pico Tag amino acid analysis system (*26*).

In Vitro Stability of ACE Inhibitory Peptide to Gastric Proteases. In vitro stability against gastric proteases was assessed by treating a 1% (w/v) peptide solution in 0.1 N HCl with pepsin for 2 h at 37 °C, the reaction was stopped by boiling in a water bath for 10 min and neutralized, and the ACE inhibitory activity was determined. An aliquot of the neutralized solution was further subjected to digestion with pancreatin (2% w/w) at 37 °C for 4 h. The ACE inhibitory activity was determined after arresting the reaction by boiling. Stability against pancreatin without prior digestion with pepsin was also evaluated.

Data Analysis. For all the inhibition assays and kinetic data, a minimum of three replicates were taken for data analysis. Using the software Origin 4.1, all the values were averaged, and the mean values were reported. Standard deviations for three replicate data were also tabulated.

RESULTS

ACE Inhibition of Glycinin Digests. The glycinin extracted from soybean meal was digested with the use of three commercially available proteases, Protease P, bovine trypsin, and chymotrypsin, and a partially purified ginger protease. ACE activity of the acetone powder extract of porcine lung and kidney was 6.7 and 5.8 units/mg, respectively. ACE inhibitory activity of the crude enzyme digest was determined (**Table 1**). The Protease P digest exhibited the most potent ACE inhibitory activity with an IC_{50} of 4.5 μg of N_2, followed by trypsin digest. The inhibitory potency toward the lung and kidney enzyme were similar (**Table 1**). The increase in the concentration of the Protease P digest to 5 μg of N_2 resulted in a sharp increase in the inhibition of both porcine lung and kidney ACE. Thereafter, the inhibition attained saturation. Digestion of glycinin with Protease P, bovine trypsin, or chymotrypsin followed by ginger protease possessing a unique specificity of cleaving C-terminal to Pro residues did not show any marked change in the IC_{50} for ACE inhibition (results not shown). The suite of peptides obtained following Protease P digestion was used in all further studies.

Isolation of ACE Inhibitory Peptides. RP-HPLC of the Protease P hydrolysate on a C-18 column resulted in five major peptide fractions with a large number of minor peptides (**Figure 1**). Among the major peptide fractions, fraction 3 exhibited the highest ACE inhibitory activity. This peptide fraction was subsequently fractionated by ion-pair chromatography using the same column and the ammonium acetate/acetonitrile solvent system. The chromatographic profile (**Figure 2**) indicated the presence of four peptides. The major peptide (peptide 3−4, **Figure 2**) eluting late in the gradient, however, did not exhibit any ACE inhibitory activity. Only peptide 3−2 (**Figure 2**) exhibited ACE inhibitory activity. This peptide fraction was further subjected to chromatography on the same column using the TFA/CH_3CN solvent system. The RP-HPLC profile shown in **Figure 3** indicated that the peptide was homogeneous. This fraction inhibited both porcine lung and kidney ACE (92% inhibition/0.04 mL). The ACE inhibitory activity of the isolated peptide was much lower than that of the synthetic ACE inhibitor captopril (IC_{50}: 0.023 mM).

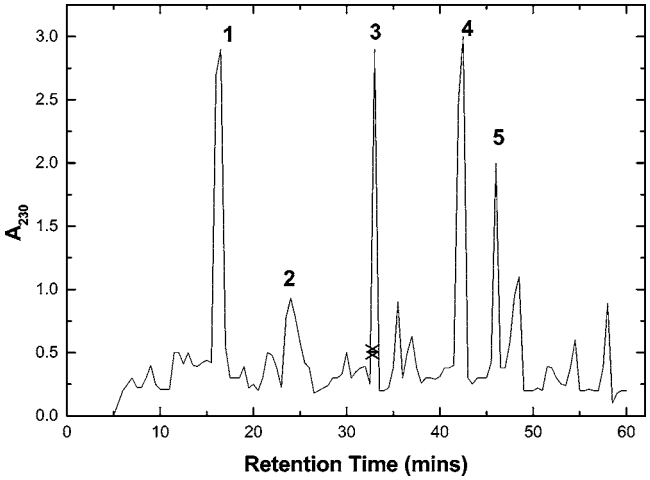

Figure 1. RP–HPLC profile of Protease P digest of glycinin. Fraction pooled indicated by an arrow.

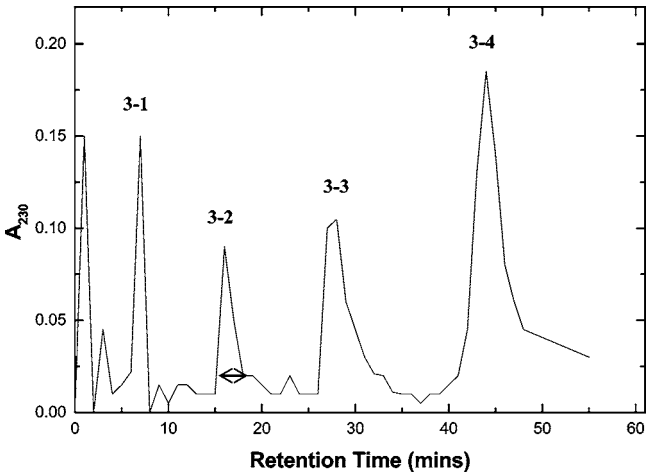

Figure 2. Ion-pair chromatography profile of peptide fraction 3 of **Figure 1**. The solvent system used was ammonium acetate/acetonitrile. Fraction pooled indicated by an arrow.

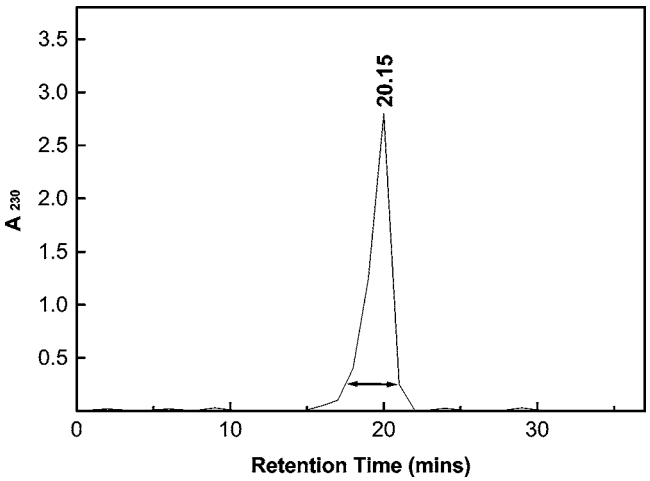

Figure 3. RP–HPLC of ACE inhibitory peptide 3–2 of **Figure 2**. Fraction pooled indicated by an arrow.

ACE Inhibition Kinetics. Inhibition kinetics of the ACE inhibitor was studied to elucidate the mechanism of action. The direct HPLC method to determine the hippuric acid released

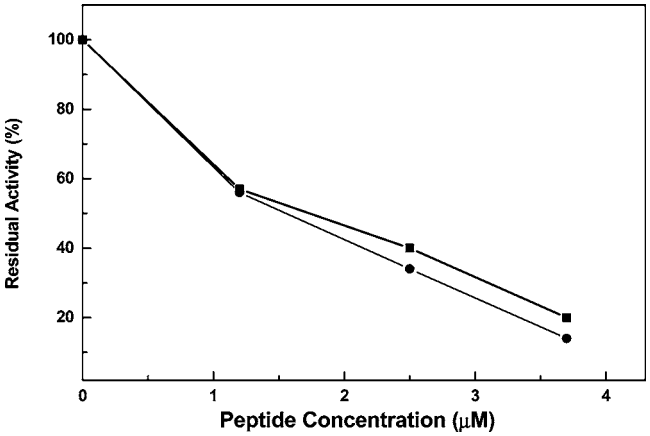

Figure 4. Determination of IC_{50} of the synthetic peptide for inhibition of porcine kidney and lung ACE. -●-, porcine lung ACE and -■-, porcine kidney ACE.

(*23*) was used to determine the initial velocity of the ACE. The amount of hippuric acid released in the presence of the fixed concentration of the purified peptide at varying substrate concentration was evaluated. The mode of inhibition was evaluated by a Lineweaver−Burk plot and found to be competitive (data not shown). The inhibition constant K_i was 4.5 μg of N_2. There was no change in the inhibitory potential of the peptide when preincubated with porcine kidney or lung ACE. The suite of peptides present in the unfractionated Protease P digest of glycinin was also competitive in nature.

Characterization of the Peptide. The amino acid composition of the purified peptide was determined after hydrolysis in vacuo with 5.8 M HCl at 110 °C for 24 h. The amino acid composition indicated the presence of valine, proline, isoleucine, and leucine. Trace amounts of serine and glycine were also detected. MALDI-TOF of the peptide indicated the presence of a single peptide of *m*/*z* 578.9. The amino terminal sequence of the peptide was determined by Edman degradation on an automated gas-phase sequencer. The sequence of the peptide was found to be NH_2-Val-Leu-Ileu-Val-Pro-COOH and corresponded to Val^{397}-Pro^{401} of the glycinin subunit G2 (Swiss Prot: P04405) of soybean (*G. max*). The calculated molecular weight of the peptide was ~540 Da. This is in close agreement with that determined by MALDI-TOF; the additional mass of 38.9 is that of K^+.

ACE Inhibitory Properties of the Synthetic Peptide. The penta peptide containing the obtained sequence VLIVP was synthesized. Synthesis of the amide form of the peptide was accomplished using Rink amide MBHA resin and FMOC strategy and purified by HPLC. A single peak was obtained, indicating that the peptide was pure. Amino terminal sequence analysis of the peptide by Edman degradation on an automated gas-phase sequenator confirmed the sequence VLIVP. The purified peptide showed the correct amino acid composition: Val (2.1), Pro (1.3), Leu (1.2), and Ile (0.9). The inhibitory potential of the synthetic peptide toward kidney ACE was evaluated as described earlier. As shown in **Figure 4**, the peptide inhibited both porcine lung and kidney ACE. The synthetic peptide was a potent inhibitor of porcine ACE with an IC_{50} of 1.69 ± 0.17 μM. The effect of inhibitor concentration on the varying substrate concentration indicated that the synthetic peptide was also a competitive inhibitor. The inhibition constant K_i calculated was $4.5 \pm 0.25 \times 10^{-6}$ M (**Figure 5**).

In Vitro Stability of Synthetic ACE Inhibitory Peptide. If these peptides are to find use as therapeutic agents, it is essential to evaluate their stability to gastric proteases. There

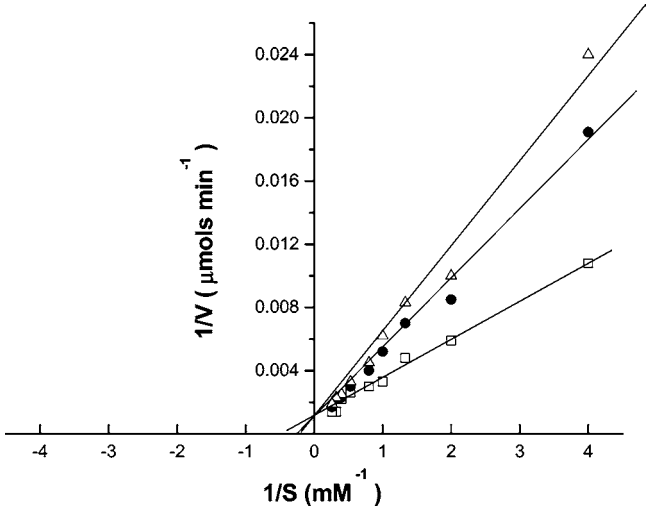

Figure 5. Lineweaver−Burk plots of porcine lung ACE inhibition by the synthetic peptide VLIVP. -□-, control; -●-, 1 μM; and -△-, 2 μM.

Table 2. In Vitro Stability of Peptide to Digestive Enzymes

enzyme	ACE inhibition (%)
None	100
pepsin (2 h, 37 °C)	100
pancreatin (4 h, 37 °C)	98
pepsin + pancreatin	96

was no change in the ACE inhibitory activity of the synthetic peptide after treatment with pepsin, pancreatin, and a combination of both (**Table 2**). The amino-terminal sequence analysis also indicated that the peptide was intact after treatment with either pepsin or pancreatin. These results demonstrate that the peptide is resistant to digestion by enzymes present in the gastrointestinal tract.

DISCUSSION

Empirically and epidemiologically there exists a relationship between eating habits and adult diseases such as cardiovascular diseases, hypertension, and diabetes. The discovery of peptides as inhibitors of ACE in food proteins has drawn considerable attention to identifying and purifying ACE inhibitors from various foods for therapeutic intervention in hypertension (*27*). Regulatory peptides encrypted in food proteins can be released from their latency by proteolytic degradation and can serve as physiological modulators of metabolism. The health-promoting properties of soybean protein and phytochemicals have been well-documented and accepted worldwide (*15*, *28*). Glycinin is the major storage protein of soybean and accounts for >25−35% of the total protein. The enzymatic hydrolysates of glycinin are potent inhibitors of ACE in vitro (**Table 1**). The hydrolysate of Protease P digestion exhibited the maximum inhibition. The IC_{50} value of this digest was 4.5 ± 0.2 μg of N_2. Although the suite of peptides obtained from bovine trypsin and chymotrypsin was also effective in inhibiting porcine ACE, increased amounts were required to bring about the same degree of inhibition (**Table 1**). The hydrolysate prepared by using two enzymes for digestion viz Protease P or trypsin followed by ginger protease did not increase the inhibitory potential toward ACE. The inhibitory activity of a chicken breast muscle extract treated with an *Aspergillus* protease was 1000-fold more potent than the untreated extract (*29*). A zein hydrolysate prepared using thermolysin exhibited hypotensive activity either on intraperitonal or oral administration to spontaneously hypertensive rats

(*10*). Soybean hydrolysates obtained from digestion with a protease from *Bacillus subtilis* have also shown a higher inhibitory effect after trypsin treatment (*16*). An array of proteases viz pepsin, trypsin, chymotrypsin, denozyme, Pronase, and actinase have been used to generate hydrolysates exhibiting ACE inhibition. An alcalase catalyzed hydrolysis of small red bean powder had a higher ACE inhibitory activity over bromelain, papain, pancreatin, trypsin, and chymotrypsin treated (*30*).

Five peptide fractions were isolated by RP-HPLC from the Protease P digest, of which fraction 3 exhibited the highest ACE inhibition. With subsequent purification by ion-pair chromatography and RP-HPLC, a single peptide fraction was isolated. Peptide fraction 3−2 (**Figure 2**) was identified as the most potent ACE inhibitory fraction. The homogeneity of the peptide was demonstrated by RP-HPLC (**Figure 3**). The sequence of this peptide was Val-Leu-Ile-Val-Pro and corresponds to segment 397−401 of glycinin subunit G2. This sequence was in close agreement to the determined amino acid composition. ACE inhibitory peptides can be classified into three groups: (a) true inhibitors, (b) ACE substrates, which are converted to inactive peptides, and (c) pro-drug peptides (*31*). Only true inhibitors and pro-drugs have the property to lower blood pressure. The peptide isolated from the Protease P digest of glycinin can be considered as a true inhibitor because the IC_{50} value for this peptide before and after preincubation with ACE were the same. This peptide of glycinin is a competitive inhibitor of both lung and porcine ACE. The chemically synthesized peptide VLIVP was a potent inhibitor of porcine lung ACE having an IC_{50} of 1.69 ± 0.17 μM and was also competitive with respect to HHL, with a K_i of $4.5 \pm 0.25 \times 10^{-6}$ M. A variety of ACE inhibitory peptides with diverse molecular properties and variable potency has been reported. A partial summary of ACE inhibitory peptides derived from food proteins with their IC_{50} values is available in a recent review by Li et al. (*32*). It has been reported that increasing the initial (uninhibited) ACE activity in an assay results in correspondingly better IC_{50} values, and this must be considered when comparing IC_{50} values from different studies (*33*). The IC_{50} of the most potent ACE inhibitory peptide (Tyr-Leu-Ala-Gly-Asn-Gln) of a soy protein peptic digest was 14 mM (*18*). The peptide Pro-Gly-Thr-Ala-Val-Phe-Lys (IC_{50}: 26.5 μM) from soy protein and the tripeptide from broccoli extract (IC_{50}: 23.7 μM) (*34*) are less potent than the peptide reported in this paper. The ACE inhibitor peptide of garlic extract (IC_{50}: 3.7 μM) (*35*), buckwheat digest (IC_{50}: 4.0 μM) (*36*), an octapeptide released from helianthinin, the 11S protein of sunflower (IC_{50}: 6.9 μM) (*37*) are in the same order if not lower than that reported in this study. However, this value is still 50-fold less potent than the synthetic ACE inhibitor captopril (IC_{50}: 0.041 μM) (*37*). The tetra-peptide VWIS obtained from the subtilisin digest of rapeseed protein is a pro-drug type of ACE inhibitor, as preincubation with ACE intensified the inhibitory activity by a factor of 6 (*14*). A tri-peptide His-His-Leu isolated from Korean soybean paste inhibits ACE, and the chemically synthesized peptide exerted ACE inhibition in vivo (*38*). The dipeptide His-Leu is released when ACE converts its physiological substrate Angiotensin I to Angiotensin II.

The structure−activity correlations between the various ACE inhibitory peptides remain ambiguous. An increasing number of sequences reported in recent years have revealed a variety of structures with inhibitory activity, and some common structure patterns have evolved. The ACE inhibitory peptide derived from glycinin, which is the major storage protein of soybean, contains Val at the amino-terminus and Pro as an imino acid at the carboxy-terminus. Cheung et al. (*39*) suggest that the most favorable amino-terminal residues are branched amino acids such as Val and Ile and that the most preferred carboxy-terminus residues are among Trp, Tyr, Pro, or Phe. The use of a ginger protease possessing a unique cleavage specificity to produce peptides with carboxy-terminal Pro residues did not increase the ACE inhibition of the Protease P digests (results not shown). Amino-terminal glycyl-dipeptides vary 300-fold in ACE inhibitory potency. Synthetic analogues of snake venom ACE inhibitor peptides containing di-carboxylic acids were also poor inhibitors of ACE (*5*). Peptides Gly-Pro-Ala-HPro, Gly-Pro-Pro-Gly-Ala (*23*), Ala-Val-Pro-Tyr-Pro-Glu-Arg (*38*), and His-His-Leu (*38*), however, do not contain branched chain amino acids at their amino-terminus and yet are potent inhibitors of ACE. The relatively high potency of the peptide isolated from glycinin could well be attributed to the presence of Val, a branched amino acid at the amino-terminus. Structure−activity correlations among peptide analogues suggested that the carboxy-terminal residues play a predominant role in competitive binding to the active center of ACE (*41*). The relatively wide substrate and inhibitor specificity of ACE can be explained by the presence of two active centers for the enzyme (*42*). In vitro gastric incubation provides easy practical mimics of the fate of such peptides after oral administration. The reduced hypotensive activity of several food-derived peptides following oral administration is due to the fact that they are hydrolyzed in the GI tract (*23*). The low susceptibility of the peptide VLIVP to hydrolysis by gastric proteases suggests compositional stability of the peptide (**Table 2**). Further studies to correlate the antihypertensive activity at the molecular level using human cell lines are in progress. The present results show that glycinin, the major storage protein of soybean that is known for its health-promoting bioactive components, is a potential starting material for enzyme-mediated production of ACE inhibitory peptides that can be used in health-enhancing functional foods.

ABBREVIATIONS USED

ACE, Angiotensin I-converting enzyme; FMOC, 9-fluorenylmethyloxycarbonyl; HBTU, 2-(^{1}H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HHL, hippuryl-histidyl-leucine; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MBHA, 4-methylbenzhydrylamine; PITC, phenylisothiocynate; PTH, phenylthiohydantoin; RP-HPLC, reverse-phase high-performance liquid chromatography; TCA, trichloroacetic acid; TFA, triflouroacetic acid.

LITERATURE CITED

(1) Laragh, J. H.; Bear, L.; Brunner, H. R.; Buhler, F. G.; Ealey, J. E.; Vaughan E. D. Renin, angiotensin, and aldosterone system in pathogenesis and management of hypertensive vascular disease. *Am. J. Med.* **1972**, *52*, 633−652.

(2) Elisseeva, Y. E.; Orekhovish, V. N.; Alexeenko, L. P.; Carboxycathepsin—a key regulatory component of two physiological systems involved in regulation of blood pressure. *Clin. Chim. Acta* **1971**, *31*, 413−419.

(3) Cushman, D. W.; Ondetti, M. A.; Gordon, E. M.; Natarajan, S.; Karanewsky, D. S.; Krapcho, J. Rational design and biochemical utility of specific inhibitors of an angiotensin-converting enzyme. *J. Cardiovasc. Pharmacol.* **1987**, *10*, 17−30.

(4) Ondetti, M. A.; Rubin, B.; Cushman, D. W. Design of specific inhibition of angiotensin-converting enzyme: new class of orally active antihypertensive agents. *Science* **1977**, *196*, 441−444.

(5) Cheung, H. S.; Cushman, D. W. Inhibition of homogeneous Angiotensin I-converting enzyme of rabbit lung by synthetic venom peptides of *Bothrops jararaca*. *Biochim. Biophys. Acta* **1973**, *293*, 451−463.

(6) Hasler, C. M. Functional foods; their role in disease prevention and health promotion. *Food Technol*. **1998**, *52*, 63−70.
(7) Clemente, A.; Vioqque, J.; Sanchez-Vioque, R.; Pedroche, J.; Bautista, J.; Milan, F. Protein quality of Chickpea (*Cicer arietinum*) protein hydrolysates. *Food. Chem.* **1999**, *67*, 269−274.
(8) Maruyama, S.; Suzuki, H. A. A peptide inhibitor of Angiotensin I-converting enzyme inhibitor derived from an enzymatic hydrolysate of casein II. Isolation and bradykinin-potentiating activity on the uterus and ileum of rats. *Agric. Biol. Chem.* **1985**, *49*, 1405−1409.
(9) Kohama, Y.; Matsumoto, S.; Oka, H.; Teramoto, T.; Okabe, M.; Mimura, T. Isolation of Angiotensin-converting enzyme inhibitor from tuna muscle. *Biochem. Biophys. Res. Commun.* **1998**, *155*, 332−337.
(10) Miyoshi, S.; Kaneko, T.; Ishizawa, Y.; Fukui, F.; Tanka, H.; Maruyama, S. Structures and activities of Angiotensin-converting enzyme inhibitors in α-zein hydrolysate. *Agric. Biol. Chem.* **1991**, *55*, 1313−1318.
(11) Okamoto, A.; Hanagata, H.; Matsumoto, E.; Kawamura, Y.; Koizumi, Y.; Yanagida. Angiotensin I-converting enzyme inhibitory activities of various fermented foods. *Boisci. Biotechnol. Biochem.* **1995**, *59*, 1147−1149.
(12) Gobbetti, M.; Ferranti, P.; Samacchi, E.; Goffredi, F. Production of Angiotensin I-converting enzyme inhibitory peptides in fermented milks started by *Lactobacillus delbruekii* subspecies *bulgaricus* SS1 and *Lactococcus lactis* subspecies *cremoris* FT5. *Appl. Environ. Microbiol.* **2000**, *66*, 3898−3904.
(13) Lee, D. H.; Kim, J. H.; Park, J. S.; Choi, Y. J.; Lee, J. S. Isolation and characterization of a novel Angiotensin I-converting enzyme inhibitory peptide derived from the edible mushroom *Tricholoma giganteum*. *Peptides* **2004**, *25*, 621−627.
(14) Marczak, E. D.; Usui, H.; Fujita, H.; Yang, Y.; Yokoo, M.; Lipkowski, A. W.; Yoshikawa, M. New antihypertensive peptides isolated from rapeseed. *Peptides* **2003**, *24*, 791−798.
(15) Potter, S. M. Overview of proposed mechanism for the hypocholesterolemic effect of soy. *J. Nutr*. **1995**, *126*, 3063−3068.
(16) Ahm, S. W.; Kim, K. M.; Yu, K. W.; Noh, D. O.; Suh, H. J. Isolation of Angiotensin I-converting enzyme inhibitory peptide from soybean hydrolysate. *Food. Sci. Biotechnol.* **2000**, *9*, 378−381.
(17) Yu, R.; Park, S.-A.; Chung, D. K.; Nam, H. S.; Shin, Z.-I. Effect of soybean hydrolysate on hypertension in spontaneously hypertensive rats. *J. Korean. Soc. Food. Sci. Nutr*. **1996**, *25*, 1031−1036.
(18) Chen, J.; Okada, T.; Muramoto, K.; Suetsuna, K.; Yang, S. Identification of Angiotensin I-converting enzyme inhibitory peptides derived from the peptic digest of soybean protein. *J. Food Biochem*. **2003**, *26*, 543−554.
(19) Chen, J.; Yang, S.; Suetsuna, K.; Chao, J. C. J. Soybean protein-derived hydrolysate affects blood pressure in spontaneously hypertensive rats. *J. Food Biochem*. **2004**, *28*, 61−73.
(20) Thompson, E. H.; Wolf, I. D. Ginger Rhizome: A new source of proteolytic enzyme. *J. Food Sci.* **1973**, *38*, 652−653.
(21) Appu Rao, A. G.; Rao, N. A method for isolation of 2S, 7S, and 11S proteins from soybeans. *Prepr. Biochem.* **1977**, *7*, 90−101.
(22) Cushman, D. W.; Cheung, H. S. Spectrophotometric assay and properties of the Angiotensin I-converting enzyme of rabbit lung. *Biochem. Pharmacol.* **1971**, *20*, 1637−1648.
(23) Wu, J.; Ding, X. Characterization of inhibition and stability of soy protein-derived Angiotensin I-converting enzyme inhibitory peptides. *Food. Res. Intl.* **2002**, *35*, 367−375.
(24) Association of official analytical chemists (AOAC) *Official methods of analysis of AOAC international*, 17th ed.; AOAC International: Arlington, VA, 2000; Method 988.05.
(25) Lineweaver, H.; Burk, D. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **1934**, *56*, 658−661.
(26) Bidlingmeyer, B. A.; Cohen, S. A.; Tarvin, T. L.; Frost, B. A new, rapid, high-sensitivity analysis of amino acids in food type samples. *J. Assoc. Off. Anal. Chem.* **1987**, *70*, 241−247.
(27) Ariyoshi, Y. Angiotensin I-converting enzyme inhibitors derived from food proteins. *Trends. Food. Sci. Technol.* **1994**, *4*, 139−144.
(28) Setchell, K. D. R. Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *J. Clin. Nutr*. **1998**, *68*, 1333s−1346s.
(29) Saiga, A.; Okumara, T.; Makhara, T.; Katsuta, S.; Shimizu, T.; Yamada, R.; Nishimura, T. Angiotensin-converting enzyme inhibitory peptides in a hydrolyzed chicken breast muscle extract. *J. Agric. Food Chem.* **2003**, *5*, 1741−1745.
(30) Kwon, Y. S.; Lee, H. G.; Shin, H. K.; Yang, C. B. Purification and identification of Angiotensin I-converting enzyme inhibitory peptide from small red bean protein hydrolysate. *Food. Sci. Biotechnol.* **2000**, *9*, 292−296.
(31) Fujita, H.; Yoshikawa, M. LKPNM: a prodrug-type ACE-inhibitory peptide derived from fish protein. *Immunopharmacology* **1999**, *44*, 123−127.
(32) Li, G.; Le, G.; Shi, Y.; Shrestha, S. Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacolgical effects. *Nutr. Res.* **2004**, *24*, 469−486.
(33) Murray, B. A.; Walsh, D. J.; Fitzgerald, R. J. Modification of the furanacryloyl-L-phenylalanylglycylglycine assay for determination of Angiotensin I-converting enzyme inhibitory activity. *J. Biochem. Biophys. Methods* **2004**, *59*, 127−137.
(34) Lee, J.; Bae, I. Y.; Lee, H. G.; Yang, C. Tyr-Pro-Lys, an Angiotensin I-converting enzyme inhibitory peptide derived from broccoli (*Brassica oleracea Italica*). *Food Chem.* **2006**, *99*, 143−148.
(35) Suetsuna, K. Isolation and characterization of Angiotensin I-converting enzyme inhibitor dipeptides derived from *Allium sativum* L. (garlic). *J. Nutr. Biochem*. **1998**, *9*, 415−419.
(36) Li, C. H.; Matsui, T.; Matsumoto, K.; Yamasaki, R.; Kawasaki, T. Latent production of Angiotensin I-converting enzyme inhibitors from buckwheat protein. *J. Peptide Sci*. **2002**, *8*, 267−274.
(37) Megias, C.; Yust, M. M.; Pedroche, J.; Lquari, H.; Giron-Calle, J.; Alaiz, M.; Millan, F.; Vioque, J. Purification of an ACE inhibitory peptide after hydrolysis of sunflower (*Helianthus annuus* L.) protein isolates. *J. Agric. Food Chem*. **2004**, *52*, 1928−1932.
(38) Shin, Z.-I.; Yu, R.; Park, S.-A, Chung, D. K.; Ahn, C. W.; Nam, H. S.; Kim, K. S.; Lee, H. J. His-His-Leu, an Angiotensin I-converting enzyme inhibitory peptide derived from Korean soybean paste, exerts antihypertensive activity in vivo. *J. Agric. Food Chem.* **2001**, *49*, 3004−30090.
(39) Cheung, H. S.; Wang, F. L.; Ondetti, M. A.; Sabo, E. A.; Cushman, D. W. Binding of peptide substrates and inhibitors of ACE. Importance of the COOH-terminal dipeptide sequence. *J. Biol. Chem.* **1980**, *255*, 401−407.
(40) Yamamoto, N.; Akino, A.; Takano, T. Antihypertensive effect from the peptides derived from the casein by an extracellular proteinase from *Lactobacillus helveticus* CP790. *J. Dairy Sci.* **1994**, *77*, 917−922.
(41) Cheung, H.; Wang, F.; Ondetti, M. A.; Sabo, E. F.; Cushman, D. W. Binding of peptide substrates and inhibitors of Angiotensin-converting enzyme. *J. Biol. Chem*. **1980**, *255*, 401−407.
(42) Perich, R. B.; Jackson, B.; Rogerson, F. M.; Mendelsohn, F. A. O.; Johnston, C. I. Two binding sites on Angiotensin-converting enzyme evidence from radio-ligand binding studies. *Mol. Pharmacol.* **1992**, *42*, 280−293.

Received for review January 27, 2006. Revised manuscript received April 13, 2006. Accepted May 1, 2006.

JF060264Q