Angiotensin I Converting Enzyme Inhibitory Peptides Purified from Bovine Skin Gelatin Hydrolysate

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Bovine skin gelatin was hydrolyzed with sequenial protease treatments in the order of Alcalase, Pronase E, and collagenase using a three-step ultrafiltration membrane reactor. The molecular weight distributions of the first, second, and third hydrolysates were 4.8–6.6, 3.4–6.6, and 0.9–1.9 kDa, respectively. The angiotensin I converting enzyme (ACE) inhibitory activity of the third hydrolysate (IC_{50} = 0.689 mg/mL) was higher than that of the first and second hydrolysates. Two different peptides showing strong ACE inhibitory activity were isolated from the hydrolysate using consecutive chromatographic methods including gel filtration chromatography, ion-exchange chromatography, and reversed-phase high-performance liquid chromatography. The isolated peptides were composed of Gly-Pro-Leu and Gly-Pro-Val and showed IC_{50} values of 2.55 and 4.67 μM, respectively.

INTRODUCTION

Food researchers have extensively studied peptides derived from protein hydrolysates as potential nutraceuticals in relation to the development of functional foods for many years (*1–3*). Among these, the new relationship between food and health has recently drawn considerable attention, and the interest is the physiological functions of some food components against certain ailments. Hypertension is a worldwide problem of epidemic proportions, which presents in 15–20% of all adults. It is the most common serious chronic health problem because it carries with it a high risk of cardiovascular complication (*4*). It is suggested that hypertension is closely related to food components, and the antihypertensive peptides may be associated with the presence of an antihypertensive peptide motif.

The molecular size of the hydrolyzed protein and the specificity of the enzyme are key factors in producing protein hydrolysates with desired functional properties. One method of controlling molecular size is the use of membrane reactors (*5, 6*). Concurrent with research aimed at establishing the use of these components in emerging functional proteins, the design and development of a process for their continuous production has received considerable attention from the protein industry. A number of functional peptides such as peptides promoting calcium absorption (*7*), reducing blood pressure (*8, 9*), and regulating cholesterol level in serum (*10*) have been isolated from milk and soybean hydrolysates.

Angiotensin I converting enzyme (ACE, dipeptidyl-carboxypeptidase) plays an important physiological role in regulating blood pressure (*11*). ACE converts an inactive form of decapeptide, angiotensin I, to a potent vasoconstrictor, octapeptide angiotensin II, and inactivates bradykinin, which has a depressor action. Following the discovery of captopril, hundreds of potential ACE inhibitors have been synthesized and at least three dozen have been tested clinically. More than a dozen ACE inhibitors have been used extensively in the treatment of essential hypertension and heart failure in humans; these include alacepril, benazepril, captopril, cilazapril, enalapril, fosinopril, lisinopril, moexipril, perindopril, quinapril, ramipril, tandolapril, and zofenopril (*12, 13*).

Many ACE inhibitory peptides have recently been isolated from various protein hydrolysates such as cheese whey (*14*), casein (*15–17*), zein (*18, 19*), soy sauce (*20*), and soybean (*21*) and also from fish muscle such as sardine (*22*), tuna (*23*), and bonito (*24*).

In the present study, our objective was to isolate ACE inhibitory peptides derived from bovine skin gelatin hydrolysates and to characterize the isolated peptides with respect to ACE inhibitory activity.

MATERIALS AND METHODS

Materials. Bovine skin gelatin was provided by Kyung-Gi Gelatin Co. (Inchon, Korea). It was stored at −35 °C and used for producing enzymatic hydrolysate. ACE (from rabbit lung) and substrate peptide (hippuryl-histidyl-leucine), trypsin (from bovine pancreas, type II), α-chymotrypsin (from bovine pancrease, type II), and Pronase E (from *Streptomyces griseus*, type XIV) were purchased from Sigma Chemical Co. (St. Louis, MO). Alcalase and Neutrase were purchased from Novo Co. (Novo Nordisk, Bagsvaerd, Denmark). The membrane reactor system for the preparation of hydrolysates was purchased from A/G Technology Co. (Needham, MA). All other reagents used in this study were reagent grade chemicals.

Preparation of Gelatin Hydrolysates. For digestion of bovine skin gelatin, five proteases, namely, Alcalase, α-chymotrypsin, Neutrase, Pronase E, and trypsin, were used. Gelatin (1 g) was dissolved in a 0.1 M sodium phosphate buffer

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Table 1. ACE Inhibitory Activity[a] of Bovine Skin Gelatin Hydrolysates Following Different Incubation Times with Various Enzymes

enzyme	$IC_{50}{}^{b}$ (mg/mL)				
	1 h	2 h	6 h	12 h	24 h
Alcalase	1.048	0.923	0.835	1.046	1.125
α-chymotrypsin	0.759	1.423	1.317	1.845	4.384
Neutrase	1.492	1.101	0.915	0.956	0.933
Pronase E	1.296	1.013	0.802	1.370	0.912
trypsin	1.046	1.040	1.044	1.159	1.130

[a] ACE inhibition was determined with 50 μL of each hydrolysate solution, 5 mg/mL. [b] IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE inhibitory activity.

Table 2. ACE Inhibitory Activity of Various Hydrolysates Prepared by Combination of Two Proteases Incubated for 6 h

enzyme[a]	$IC_{50}{}^{b}$ (mg/mL)
α-chymotrypsin → Alcalase	1.479
neutrase → Alcalase	1.423
Pronase E → Alcalase	0.969
trypsin → Alcalase	0.962
Alcalase → Pronase E	0.593
Alcalase + Pronase E	0.642

[a] →, addition order; +, addition at the same time. [b] IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE inhibitory activity.

solution, hydrolyzed with the protease at a ratio of protein to enzyme of 100:1 (w/w) for 6 h in a batch reactor, and then heated at 98 °C for 10 min to inactivate the proteases. The resulting hydrolysate was assayed for in vitro ACE inhibitory activity.

A three-step ultrafiltration membrane reactor (*25*) was used for the preparation of enzymatic hydrolysates of bovine skin gelatin. In the first step of the membrane reactor, 1% (w/v) gelatin was digested with Alcalase (ratio of protein to enzyme of 50:1, w/w) at pH 8.0 and 50 °C and fractionated through a membrane (model UFP-10-C-4, A/G Technology Co.) with a 10 kDa molecular weight cutoff (MWCO), followed by hydrolysis with Pronase E (ratio of protein to enzyme of 33:1, w/w) at pH 8.0 and 50 °C. The resultant hydrolysate was fractionated through a membrane (model UFP-5-C-4, A/G Technology Co.) with a 5 kDa MWCO and, finally, hydrolyzed with collagenase (ratio of protein to enzyme of 100:1, w/w) at pH 7.5 and 37 °C. The resultant hydrolysate was separated through a membrane (model UFP-1.0-43-PM1, Romicon, Cummings Park, Woburn, MA) with a 1 kDa MWCO, followed by lyophilization and subsequent storage at −20 °C until use.

Measurement of ACE Inhibitory Activity. The ACE inhibitory activity assay was performed using a modified version of the method of Cushman and Cheung (*26*). A sample solution (50 μL) with 50 μL of ACE solution (25 milliunits/mL) was preincubated at 37 °C for 5 min and then incubated with 150 μL of substrate (8.3 mM Hip-His-Leu in 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3) at 37 °C for 60 min. The reaction was stopped by the addition of 250 μL of 1 M HCl. The resulting hippuric acid was extracted with 1.5 mL of ethyl acetate. After centrifugation (800*g*, 15 min), 1 mL of the upper layer was transferred into a glass tube and evaporated at room temperature for 2 h in a vacuum. The hippuric acid was dissolved in 3.0 mL of distilled water, and the absorbance was measured at 228 nm using a Hitachi model U-3210 (Hitachi, Tokyo, Japan) spectrophotometer. The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE inhibitory activity.

Molecular Weight Distribution of Hydrolysates. The molecular weight distribution of the hydrolysates was determined by gel filtration chromatography using a Sephadex G-25 column (2.5 × 98 cm). A 0.1 M phosphate buffer (pH 7.0) was used in this experiment. Chromatography was carried out at room temperature with a flow rate of 0.5 mL/min, and A_{280}
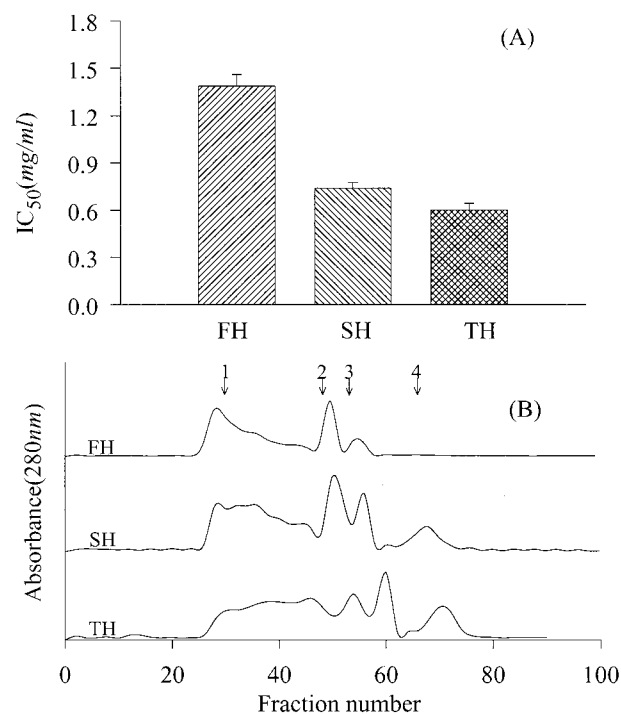

Figure 1. ACE inhibitory activity (A) and molecular weight distribution profiles (B) of gelatin hydrolysates prepared by three-step ultrafiltration membrane reactor. The following molecular weight standards were used: 1, insulin (MW = 5700 Da); 2, gramicidin D (MW = 2000 Da); 3, vitamin B_{12} (MW = 1355 Da); 4, tetraphenylalanine (MW = 667 Da). FH, first-step hydrolysates; SH, second-step hydrolysates; TH, third-step hydrolysates.

was measured. Two milligrams of each hydrolysate was applied onto the column. A standard distribution was determined by chromatographing several standards, namely, insulin, gramicidin, vitamin B_{12}, and tetraphenylalanine.

Amino Acid Analysis of Hydrolysates. The hydrolysates (50 mg) were hydrolyzed for 24 h in 6 M HCl containing 0.1% thioglycolic acid at 110 °C under vacuum. Amino acids were derivatized with phenylisothiocyanate followed by identification and quantification using an automatic amino acid analyzer (Biochrom 20, Pharmacia Biotech, Cambridge, U.K.).

Purification of ACE Inhibitory Peptide. The hydrolysate (0.1 g) was fractionated using a Sephadex G-25 column (2.5 × 90 cm) and subsequently loaded onto an SP-Sephadex C-25 (2.5 × 45 cm), previously equilibrated with 20 mM sodium acetate/acetic acid buffer (pH 4.0). The separation was performed with a linear gradient from 0 to 1.0 M NaCl in the same buffer. Peptides in the eluate, in which the ACE inhibitory activity was detected, were pooled, concentrated by ultrafiltration, dialyzed, lyophilized, and then separated by reverse-phase high-performance liquid chromatography (RP-HPLC, P2000, Sepctra-Physics, San Jose, CA) using a Primesphere ODS C_{18} column (10 × 250 mm, Phenomenex, Cheshire, U.K.). The separation was performed with a linear gradient from 10 to 50% in acetonitrile containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 2 mL/min.

The peptides showing ACE inhibitory activity were further purified using capillary electrophoresis (BioFocus 2000, Bio-Rad Co., Cambridge, MA) with a coated column (24 cm × 25 μm, Bio-Rad Co.) in 0.1 M phosphate buffer solution (pH 2.5) at 10.0 kV. Absorbance was monitored at 200 nm for the detection of peptides.

Amino Acid Sequences of Peptides. The purified peptides showing ACE inhibitory activity were further subjected to the automated Edman degradation on a Perkin-Elmer (model 470A, Applied Biosystem Division, Branchburg, NJ) protein sequencer according to the manufacturer's instructions.
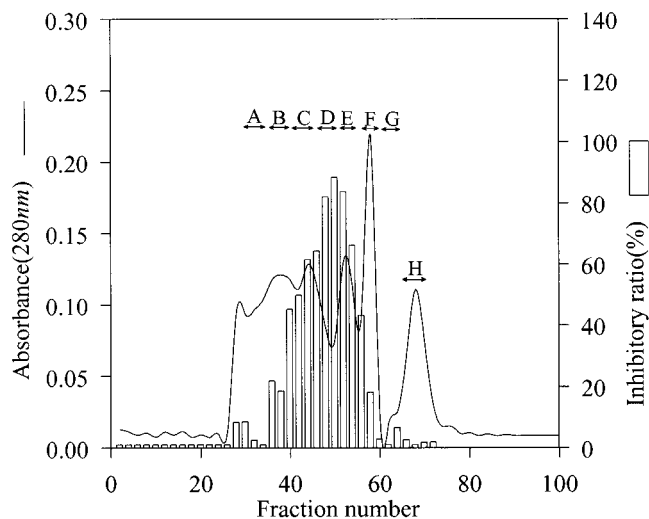
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Figure 2. Gel filtration chromatography of the third hydrolysates on the Sephadex G-25 column. Separation was performed at a flow rate of 0.5 mL/min and collected at a fraction volume of 5 mL. Aliquots, drawn from each fraction, were used to measure ACE inhibitory activity (solid bar). The fraction showing proteins and ACE inhibitory activity were designated A–H.

RESULTS AND DISCUSSION

Preparation of Enzymatic Hydrolysates. As summarized in Table 1, the overall catalytic activity of ACE inhibitory peptides was increased after digestion with various proteolytic enzymes. To select suitable proteases for gelatin hydrolysis, gelatin extract from bovine skin was independently hydrolyzed with Alcalase, α-chymotrypsin, Neutrase, Pronase E, and trypsin using a batch reactor. Among them, the hydrolysates digested with Alcalase and Pronase E for 6 h revealed a high ACE inhibitory activity showing IC_{50} values of 0.835 and 0.802 mg/mL, respectively.

Samples of gelatin were also allowed to react with combinations of two proteases for 6 h under optimal hydrolysis conditions. High ACE inhibitory activity of hydrolysates was observed in hydrolysates prepared with a combination of Alcalase and Pronase E, and the IC_{50} value was increased slightly (Pronase E → Alcalase, 0.969 mg/mL; Alcalase → Pronase E, 0.593 mg/mL) as summarized in Table 2. Therefore, Alcalase and Pronase E were selected for effective digestion of gelatin.

Collagenase specifically hydrolyzes the X–Gly peptide bond of the sequence Pro-X-Gly-Pro. Digestion of gelatin that contains this sequence by collagenase produces peptides with a C-terminal Pro-X or N-terminal Gly-Pro sequence. Oshima et al. (*27*) reported nine ACE inhibitory peptides, ranging from tri- to dodecapeptide, that were isolated from collagenase digests of gelatin. Therefore, a three-step ultrafiltration membrane reactor was designed to perform enzymatic hydrolysis of bovine skin gelatin, which was composed of consecutive digestions with Alcalase, Pronase E, and collagenase. The hydrolysate at each step was fractionated using ultrafiltration membranes with MWCO values of 10, 5, and 1 kDa, followed by ACE inhibitory activity assays. The IC_{50} value (0.689 mg/mL) of the third hydrolysate was increased 2-fold compared to that of the first hydrolysate, which had a value of 1.407 mg/mL (Figure 1A).

The data of ultrafiltration showed that the low molecular weight fraction (<1000 Da) of bovine skin gelatin hydrolysates had a higher ACE inhibitory activity.
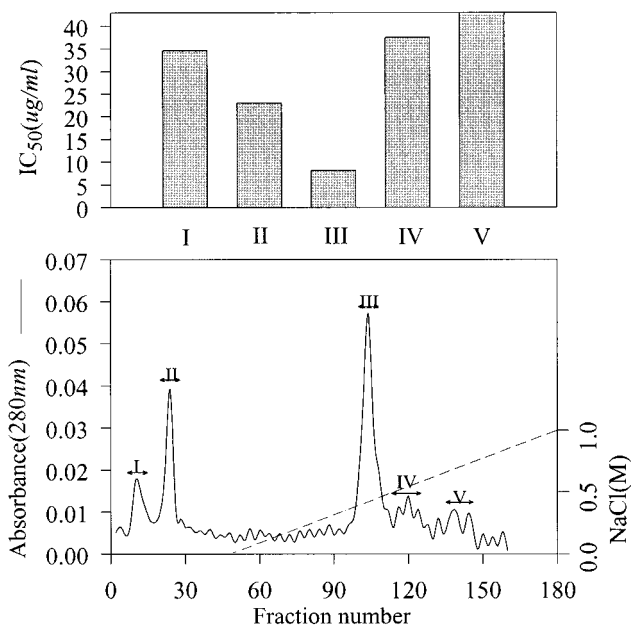

Figure 3. Separation of peptides from active fraction E isolated from the third hydrolysates using an SP-Sephadex C-25 column. Its bound peptides were eluted with a linear gradient from 0 to 1.0 M NaCl in a 20 mM sodium acetate/ acetic acid buffer (pH 4.0) at a flow rate of 2 mL/min and collected at a fraction volume of 5 mL. The fractions showing proteins and ACE inhibitory activity were designated I–V.
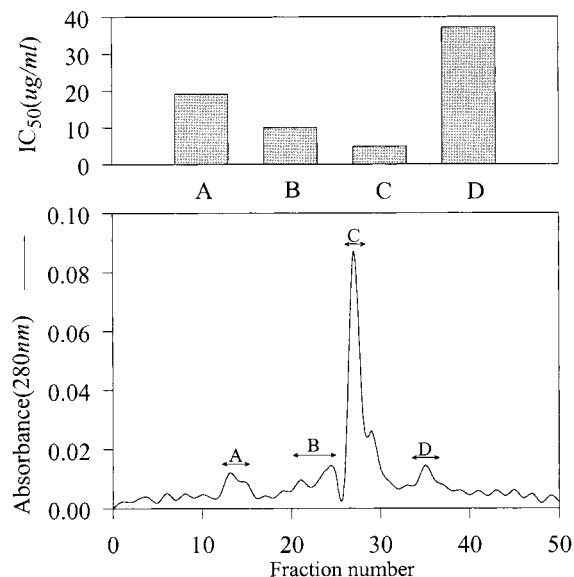

Figure 4. Separation of peptides from active fraction EIII on a Sephadex G-15 column. The fraction was eluted with distilled water at a flow rate of 0.5 mL/min and collected at a fraction volume of 5 mL. The fractions showing proteins and ACE inhibitory activity were designated A–E.

Characterization of Hydrolysates. The amino acid compositions of gelatin and hydrolysates showed nearly identical values, and glycine had the highest content of ~40% (data not shown). In addition to specific amino acid compositions, the molecular weights of the hydrolysates were critical factors in producing peptides possessing ACE inhibitory activity. One of the methods for fractionating different molecular weight hydrolysates is the use of a membrane reactor using ultrafiltration membranes with different pore sizes (*5*). However, this technique has some limitations such as permeation of
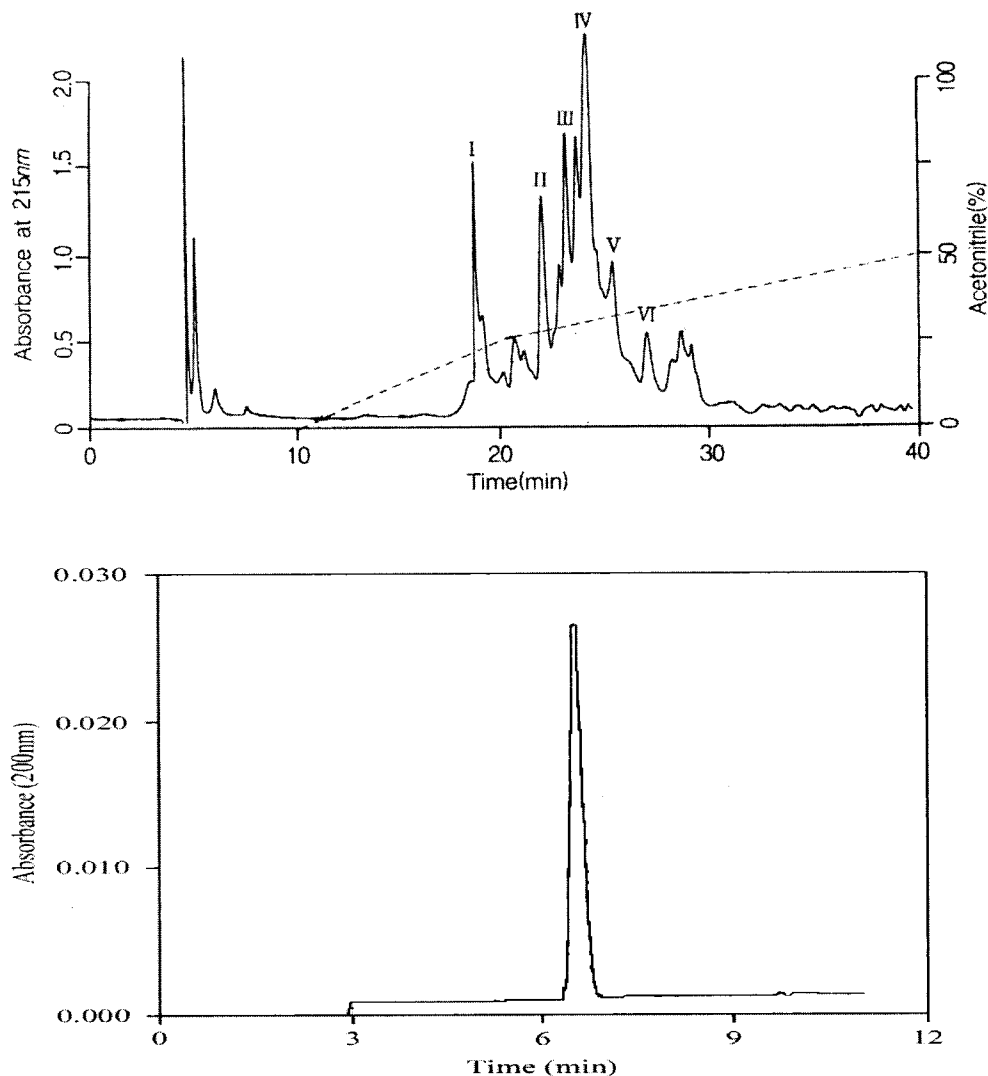
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Figure 5. Separation of the peptides from active fraction EIIIC by RP-HPLC. The separation was performed with a linear gradient from 10 to 50% in 40 min at a flow rate of 2 mL/min (A = 0.1%, B = 0.1% TFA in acetonitrile/H$_2$O). Elution was monitored at 215 nm. The fractions were designated I−VI and were assayed for ACE inhibitory activity (A). The purity of active fraction EIIICIV was confirmed by a capillary electrophoresis system. The capillary electrophoresis was performed using BioFocus 2000 with a coated column (24 cm × 25 μm) in 0.1 M phosphate buffer solution (pH 2.5) at 10.0 kV (B).

overlapping molecular weights of the hydrolysates (*28*). Various ultrafiltration membranes having broad pore sizes may solve this problem.

A membrane reactor with specific ultrafiltration membranes with different MWCO sizes was used for fractionating enzymatic hydrolysates. Finally, the molecular weight distributions of fractionated hydrolysates were determined using gel filtration. As shown in Figure 1B, the major peaks of the first-step hydrolysate were located at ~7000 Da and no peaks were seen below 600 Da, whereas the molecular weights of the second- and third-step hydrolysates were located below 600 Da. Thus, the molecular weights of the first-step hydrolysate were decreased by continuous hydrolysis in the second- and third-step ultrafiltration membrane reactor using different proteases. This observation suggests that continuous enzymatic hydrolysis with different proteases and fractionation of the resulting hydrolysates using different ultrafiltration membranes produced small molecular weight hydrolysates having ACE inhibitory activity.

Purification and Amino Acid Sequences of ACE Inhibitory Peptides. ACE inhibitory peptides were purified to homogeneity from the third-step hydrolysate

using sequential chromatographic methods. Size exclusion chromatography on a Sephadex G-25 column was used to fractionate the hydrolysates, and the flow-through fractions were pooled in order to obtain fractions A−H (Figure 2). Among the different fractions, fraction E exhibited the strongest ACE inhibitory activity. The major purification step was achieved using cation-exchange chromatography on an SP-Sephadex C-25. As depicted in Figure 3, bound peptides were eluted with a linear gradient of 0−1.0 M NaCl. There were two major peptide peaks, which were monitored at 220 nm in the elution profile of fractions from the cation-exchange column. The first peptide peak (fraction III), which eluted at 0.4−0.5 M NaCl concentration, exhibited strong ACE inhibitory activity (IC$_{50}$ = 8.75 μg/mL) and was designated fraction EIII.

Fraction EIII was further purified by gel permeation chromatography on a Sephadex G-15 column and further fractionated to five new fractions designated A−E (Figure 4). Fractions exhibiting ACE inhibitory activity were pooled (fraction C) and designated fraction EIIIC.

The active peptide fraction (EIIIC) was separated on a reversed-phase column (ODS C$_{18}$) via HPLC; the elution profiles of the peptides are shown in Figure 5A.

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Table 3. Purification of ACE Inhibitory Peptides from Bovine Skin Gelatin Hydrolysate

fraction	step	IC_{50}^a (mg/mL)
E	Sephadex G-25	436
EIII	cation exchange	8.17
EIIIC	Sephadex G-15	4.92
EIIICIII	RP-HPLC	1.50
EIIICIV	RP-HPLC	0.82

^{a} IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE inhibitory activity.

Table 4. Amino Acid Sequences of ACE Inhibitory Peptides Purified from Bovine Skin Gelatin Hydrolysates

fraction	peptide	IC_{50}^a (μM)
EIIICIII	Gly-Pro-Val	4.67
EIIICIV	Gly-Pro-Leu	2.55

^{a} IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE inhibitory activity.

Two peptide fractions exhibiting ACE inhibitory activity were separated and further purified using gel permeation chromatography through the same reversed-phase column with a different gradient. After the second chromatography, a single pure peptide was isolated and designated fractions EIIICIII and EIIICIV, respectively. The purity of each peptide was confirmed by using capillary electrophoresis (Figure 5B). Typical results obtained during the purification steps are summarized in Table 3.

The amino acid sequences and the IC_{50} values of each peptide are listed in Table 4. The resultant two ACE inhibitory peptides were composed of three amino acid residues. The observed sequences of fractions EIIICIII and EIIICIV were Gly-Pro-Val and Gly-Pro-Leu, respectively. The peptide from fraction EIIICIV shows a higher ACE inhibitory activity ($IC_{50} = 2.55$ μM) than that of fraction EIIICIII ($IC_{50} = 4.67$ μM).

Partial enzymatic hydrolysis studies of collagen have demonstrated widespread distribution of glycine and its frequent occurrence in polypeptide chains such as in Gly-Pro-X, and Gly-Pro-Hypro (*29*). Collagenase specifically hydrolyzes the X−Gly peptide bond of the sequence Pro-X-Gly-Pro and produces a Gly-Pro sequence at the N terminus or a Pro-X at the C terminus (*30*). According to previous papers, inhibitory peptides of ACE have at least one proline residue (*14, 24, 31, 32*). Similar results were observed in this study; two isolated ACE inhibitory peptides were composed of Gly-Pro at the N terminus and either Pro-Leu or Pro-Met at the C terminus.

The amino acid sequences from some proteins such as casein, fish proteins, and other proteins with high ACE inhibitory acitivities are known as dipeptide (Arg-Pro) to dodecapeptide (Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys) (*16, 33*). The peptides from the pepsin hydrolysate of sardine (Val-Lys-Ala-Gly-Phe, Lys-Val-Leu-Ala-Glu-Met, and Leu-Lys-Leu) and kidney bean also had high ACE inhibitory activities (*34, 35*).

Matsumura et al. (*24*) isolated four ACE inhibitory peptides from an autolysate of bonito bowels, and these peptides (Leu-Arg-Pro, Ile-Arg-Pro, Val-Arg-Pro, and Ile-Lys-Pro) had IC_{50} values of 1.0, 1.8, 2.2, and 2.5 μM, respectively. It should be noted that peptides derived from α-zein have been shown to have significant physiological effects and have been used in an attempt to prevent hypertension (*18, 36*). Miyoshi et al. (*18*) reported that some tripeptides isolated from a hydroly-

sate of α-zein, a maize endosperm protein, act as ACE inhibitors. These peptides, Leu-Arg-Pro, Leu-Ser-Pro, Leu-Gln-Pro, and Leu-Ala-Tyr, had IC_{50} values of 0.27, 1.7, 1.9, and 3.9 μM, respectively. They also found that Leu-Arg-Pro had a hypotensive activity, as a 30 mg/kg intravenous injection decreased the blood pressure of spontaneously hypertensive rats by 15 mmHg.

The results of this study suggest that ACE inhibitory activities derived from bovine skin gelatin hydrolysate could be utilized to develop physiologically functional foods. In addition, it is expected that increasing interest will be shown in basic research and potential applications of bioactive peptides.

ABBREVIATIONS USED

ACE, angiotensin I converting enzyme; MWCO, molecular weight cutoff; TFA, trifluoroacetic acid.

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