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Purification and Characterization of a Novel Angiotensin I-Converting Enzyme Inhibitory Peptide Derived from an Enzymatic Hydrolysate of Duck Skin Byproducts

Seung-Jae Lee,[†] Yon-Suk Kim,[†] Seong-Eun Kim,[†] Eun-Kyung Kim,[‡,⊥] Jin-Woo Hwang,[†] Tae-Kyu Park,[†] Bo Kyung Kim,[#] Sang-Ho Moon,[⊥] Byong-Tae Jeon,[⊥] You-Jin Jeon,[§] Chang-Bum Ahn,[⊗] Jae-Young Je,[⊗] and Pyo-Jam Park*,[†,⊥]

[†]Department of Biotechnology, [‡]Department of Natural Science, Division of Food Bioscience, [#]College of Medicine Institute of Biomedical Science and Technology, and [⊥]Korea Nokyong Research Center, Konkuk University, Chungju 380-701, Korea

[§]Faculty of Applied Marine Science, Jeju National University, Jeju 690-756, Korea

[⊗]Department of Food Science and Nutrition, Chonam National University, Yosu 550-749, Korea

ABSTRACT: An angiotensin I-converting enzyme (ACE) inhibitory peptide was isolated and identified from hydrolysates of duck skin byproducts. Duck skin byproducts were hydrolyzed using nine proteases (Alcalase, Collagenase, Flavourzyme, Neutrase, papain, pepsin, Protamex, trypsin, and α-chymotrypsin) to produce an antihypertensive peptide. Of the various hydrolysates produced, the α-chymotrypsin hydrolysate exhibited the highest ACE inhibitory activity. The hydrolysate was purified using fast protein liquid chromatography (FPLC) and high-performance liquid chromatography (HPLC). The amino acid sequence of the ACE inhibitory peptide was identified as a hexapeptide Trp-Tyr-Pro-Ala-Ala-Pro, with a molecular weight of 693.90 Da. The peptide had an IC_{50} value of 137 μM, and the inhibitory pattern of the purified ACE inhibitor from duck skin byproducts was determined to be competitive by Lineweaver−Burk plots. In addition, the peptide was synthesized and the ACE inhibitory activity was verified in vivo. Spontaneously hypertensive rats (SHR) exhibited significantly decreased blood pressure and heart rate after peptide injection. Taken together, the results suggest that Trp-Tyr-Pro-Ala-Ala-Pro may be useful as a new antihypertensive agent.

KEYWORDS: *antihypertensive peptide, ACE inhibitory activity, duck skin byproduct, enzymatic hydrolysis*

INTRODUCTION

Bioactive peptides isolated from natural substance proteins are potential modulators of various regulatory processes in the human body and have the potential to reduce the risk of disease.[1] Among biologically active peptides, peptides that inhibit ACE have been extensively studied.[2,3] ACE plays an important role in the regulation of blood pressure. ACE is a dipeptidyl carboxypeptidase (EC 3.4.15.1) that not only converts the decapeptide (angiotensin I) to the potent vasoconstrictive octapeptide (angiotensin II) but also inactivates the antihypertensive vasodilator bradykinin, a system that, together, increases blood pressure.[4] Recent studies have focused on the design and production of functional components from protein hydrolysates.[5,6] Since the discovery of ACE inhibitors in snake venom, many studies have attempted to synthesize ACE inhibitors, resulting in drugs such as captopril, enalapril, alacepril, and lisinopril, which are currently used for the treatment of essential hypertension and heart failure in human.[7,8] However, the use of these drugs is under strict regulation due to the potential health hazards caused by such compounds. Therefore, a significant research effort is currently directed at identifying naturally existing antihypertension compounds as replacements for synthetic antihypertension compounds. Recently, many antihypertensive peptides have been purified from various natural substances, such as cheese whey,[9] casein,[10,11] zein,[12] corn gluten,[13] and bovine blood plasma.[14] However, the antihypertensive effect of livestock products has not yet been evaluated. Duck meat and its protein are considered to be healthy because these food products have been shown to reduce blood cholesterol levels and display antioxidant activity.[15,16] In addition, these products have been used as functional foods with cardiovascular benefits. The properties of duck meat are unlike those of other animals in that it is alkaline, which helps slow the aging process of prepared foods.[17]

In the present study, we identified an antihypertensive peptide from duck skin byproducts. Almost all of the natural hypotensive ACE inhibitors were peptides that displayed high competitive affinity with the ACE active site. Therefore, the objectives of this study were to isolate the ACE inhibitory compounds from duck skin byproducts and to identify a new ACE inhibitory peptide.

MATERIALS AND METHODS

Materials. Duck skin byproducts were obtained from a local duck farm (Eumseong, Korea). ACE (EC 3.4.15.1) from rabbit lung, hippuryl-histidyl-leucine (HHL) as a substrate peptide of ACE, proteases, papain, pepsin, α-chymotrypsin, and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). Four enzymes,

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Flavourzyme, Neutrase, Protamex, and Alcalase, were obtained from Novo Co. (Novozyme, Nordisk, Bagsvaerd, Denmark). All other reagents were of the highest grade commercially available.

Preparation of Enzymatic Hydrolysates from Duck Skin Byproducts. Prior to enzymatic hydrolysis, duck skin byproducts were pulverized into powder using a grinder after fat removal by 0.1 M acetic acid treatment. Briefly, 1000 mL of buffer solution was added to 1.0 kg of powdered sample, and then 20 mL (or g) of each enzyme (Alcalase, EC 3.4.21.62; Collagenase, EC 3.4.24.7; Flavourzyme, EC 3.4.11.1; Neutrase, EC 3.4.24.4; papain, EC 3.4.22.2; pepsin, EC 3.4.23.1; Protamex, EC 3.4.21.14; trypsin, EC 3.4.21.4; and α-chymotrypsin, EC 3.4.21.1) was added after preincubation for 30 min. The enzymatic hydrolysis reactions were performed for 8 h to achieve an optimum hydrolytic level and immediately heated to inactivate the enzyme at 100 °C for 10 min. The mixture was then rapidly cooled to 20−25 °C in an ice bath. The hydrolysates recovered were lyophilized in a freeze-dryer for 3 days.[18,19]

ACE Inhibitory Activity. A sample solution (50 μL) with 50 μL of ACE solution (25 milliunits/mL) was preincubated at 37 °C for 5 min, and then the mixture was incubated with 150 μL of substrate (8.3 mM HHL in 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3) for 60 min at the same temperature. The reaction was terminated 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 to a test 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 UV spectrophotometer (Varian Inc., Australia).[20]

Separation by Tangential Flow Filtration (TFF). The molecular weight of α-chymotrypsin hydrolysate was determined in our previous study.[21] The α-chymotrypsin hydrolysate solution was passed through ultrafiltration membranes (5, 10, and 30 kDa) using a Millipore Labscale TFF system (Millipore Corp., Bedford, MA, USA) to obtain four distinct molecular weight fractions. All fractions recovered were lyophilized in a freeze-dryer for 3 days.

Liquid Chromatography Using a C_{18} and GPC Column. The α-chymotrypsin hydrolysate that exhibited the highest antihypertensive activity was further purified using FPLC on a GPC column (HiPrep 26/60 Sephacryl S-100 HR) at a flow rate of 2.0 mL/min. The elution peaks were detected at 240 nm, concentrated using a rotary evaporator, and lyophilized for 3 days. For further purification, the fraction with the highest ACE inhibitory activity from reverse-phase HPLC (RP-HPLC) on a C_{18} column (250 × 20 mm) with a linear gradient of acetonitrile (0−80%) at a flow rate of 2.0 mL/min was determined. The elution peaks were detected at 215 nm, concentrated using a rotary evaporator, and lyophilized for 3 days. Further isolation of the fraction with the highest ACE inhibitory activity from RP-HPLC was loaded onto a C_{18} column (250 × 4.6 mm) with a linear gradient of acetonitrile (0−70%) at a flow rate 0.5 mL/min. Potent peaks were collected, evaluated for antihypertensive activity, and then lyophilized. The fraction that showed the highest ACE inhibitory activity was further purified over a GPC SB-802.5 column (8.0 × 300 mm) at a flow rate 0.1 mL/min. This same fraction was then applied onto a GPC SB-803 column (8.0 × 300 mm) at a flow rate of 0.05 mL/min.[16] The amino acid sequence of the final purified peptide was analyzed.

Identification of the Amino Acid Sequence of the Purified Peptides. Accurate molecular mass and amino acid sequence of the purified peptide was determined using a GC/LC triple-quadrupole mass spectrometer (Shinhan Scientific) at the Korea Basic Science Institute. The purified peptide was searched for in the NCBI nonredundant peptide database.

Synthesis and Purification of Peptides. Peptides were synthesized by Fmoc solid phase peptide synthesis (SPPS) using ASP48S (Peptron Inc.) and purified by the RP-HPLC using a Vydac Everest C_{18} column (250 mm × 22 mm, 10 μm). Elution was carried out with a water/acetonitrile linear gradient (3−40% (v/v) of acetonitrile) containing 0.1% (v/v) trifluoroacetic acid. The molecular weight of the purified peptide was confirmed using LC-MS (Agilent HP1100 series).

Determination of the Inhibition Pattern on ACE. The enzyme activity was measured at different substrate concentrations. The kinetics of ACE in the presence of the inhibitor was determined using Lineweaver−Burk plots.[22]

Animals and Measurement of Blood Pressure and Heart Rate. Adolescent (8-week-old) male SHR and normotensive rats (Wistar) were purchased from Orient Bio (Seongnam, Korea). Animals were maintained on a 12 h light/dark cycle and given access to standard laboratory chow and distilled water ad libitum. After 1 week of acclimation in the Konkuk University Animal Research Center, SHRs were randomly assigned to untreated or antihypertensive peptide (synthetic peptide) and captopril-treated groups. The rats were then challenged with an intravenous injection of captopril at a dosage of 10 mg/kg body weight, and the synthesis peptide was injected at a dose of 1.0 mg/kg body weight.[23] Normotensive rats were administered the same volume (1.0 mg/kg) of saline solution. The heart rate was measured on the basis of beat-to-beat intervals. Systolic blood pressure was measured using the tail cuff method (UR-5000, Ueda Co. Ltd., Tokyo, Japan) after the rats were warmed in a 40 °C chamber for 10 min.

Statistical Analysis. Data were evaluated for statistical significance using the SPSS package for Windows. Values were expressed as the mean ± standard error (SE). The mean values were compared using a one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test to determine differences between the means of the treatments, with significance at $p < 0.05$.

RESULTS AND DISCUSSION

In our previous study, antihypertensive inhibitory activity of the hydrolysates was evaluated using an ACE inhibitory assay.[24] Among the hydrolysates tested, the highest ACE inhibitory activity was observed in the α-chymotrypsin hydrolysates, exhibiting an IC_{50} value of 1.724 mg/mL (Table 1). For this reason, the α-chymotrypsin hydrolysate was selected for further study.

Table 1. ACE Inhibitory Activity and Purification Fold during the Stages of Purification from the Duck Skin Byproduct

fraction	ACE inhibitory activity (IC_{50}, mg/mL)	purification (fold)	recovery rate (%)
α-chymotrypsin hydrolysate	1.724	1.0	100.0
FPLC (size exclusion)	0.591	1.09	12.032
first HPLC (250 × 200 mm)	0.282	1.83	4.071
second HPLC (250 × 4.6 mm)	0.236	4.72	0.093
third HPLC (300 × 8 mm)	0.109	15.06	0.044
fourth HPLC (300 × 8 mm)	0.095	23.56	0.018

To purify the antihypertensive peptide, the α-chymotrypsin hydrolysate of duck skin byproducts was filtered through 5, 10, and 30 kDa membrane filters. The yields for <5, 5−10, 10−30, and >30 kDa were 42.0, 12.7, 9.8, and 33.9%, respectively (data not shown). The lowest molecular weight fraction (<5 kDa molecular weight cutoff (MWCO)) in the TFF system of the duck skin byproduct protein had higher ACE inhibitory activity than the higher molecular weight fractions, exhibiting an IC_{50} value of 0.816 mg/mL (data not shown). Many studies have reported that short peptides exert greater antioxidant potential

and other bioactive properties as compared to the longer peptides.[25] Therefore, the fraction obtained from the 5 kDa MWCO was selected for further study. This lyophilized fraction was further separated by FPLC on a GPC column (HiPrep 26/60 Sephacryl S-100 HR) using distilled water, and the elution was separated into eight fractions (A, B, C, D, E, F, G, and H). The fractions were pooled and lyophilized. Among all fractions collected, fraction E exhibited the strongest ACE inhibitory activity (Figure 1). This lyophilized fraction was further isolated

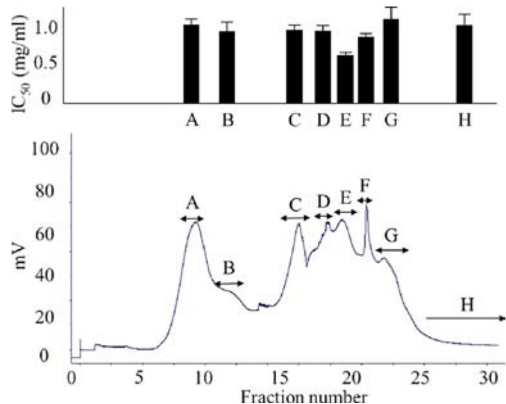

Figure 1. Purification profiles of antihypertensive peptides from duck skin byproducts: FPLC trace on a GPC column (HiPrep 26/60 Sephacryl S-100 HR) of active fraction E eluted on the FPLC (lower panel) and ACE inhibitory activity (upper panel) of the fractions. FPLC operation was carried out with distilled water at a flow rate of 4.0 mL/min using a UV detector at 240 nm.

by RP-HPLC on a C_{18} column (250 × 20 mm) using a gradient of acetonitrile (0−80%), and the elution was separated into five fractions (E-I, E-II, E-III, E-IV, and E-V). The lyophilized fraction E-I, which had the highest ACE inhibitory activity (Figure 2), was subjected to a second RP-HPLC step on a C_{18} column (250 × 4.6 mm) using a gradient of acetonitrile (0−70%) and fractionated into four portions. The fractions were pooled and lyophilized. Among all fractions collected, fraction E-I-ii exhibited the strongest ACE inhibitory activity (Figure 3). To obtain a purified peptide, E-I-ii was rechromatographed

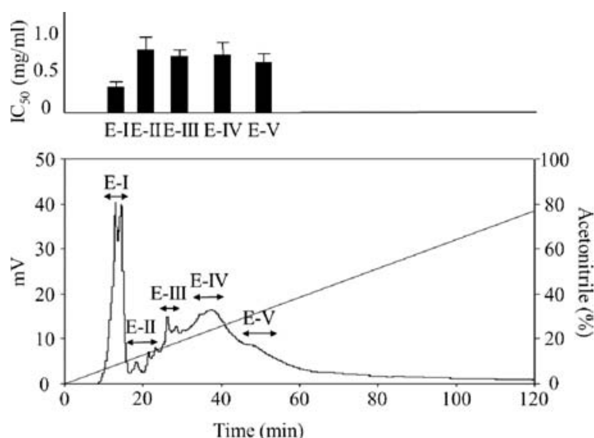

Figure 2. Reversed-phase HPLC trace on a C_{18} column (250 × 20 mm) of active fraction E-I eluted on the HPLC (lower panel) and ACE inhibitory activities (upper panel) of the fractions. HPLC operation was carried out with a linear acetonitrile gradient (0−80%) at a flow rate of 4.0 mL/min using a UV detector at 215 nm.

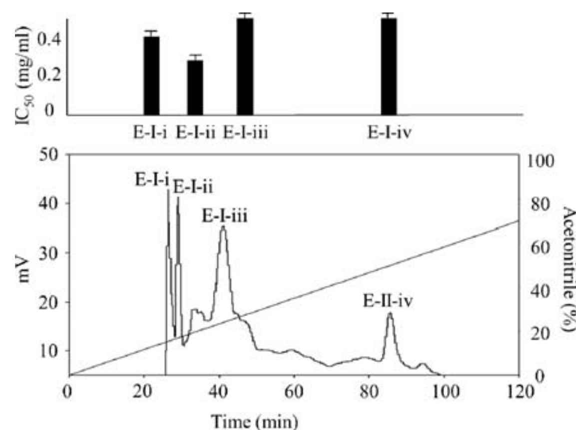

Figure 3. Reversed-phase HPLC trace on a C_{18} column (250 × 4.6 mm) of active fraction E-I-ii eluted on the HPLC (lower panel) and ACE inhibitory activities (upper panel) of the fractions. HPLC operation was carried out with a linear acetonitrile gradient (0−70%) at a flow rate of 1.0 mL/min using a UV detector at 215 nm.

through a third RP-HPLC step on a GPC SB-802.5 column (300 × 8.0 mm) (Figure 4). For this step, of the fractions

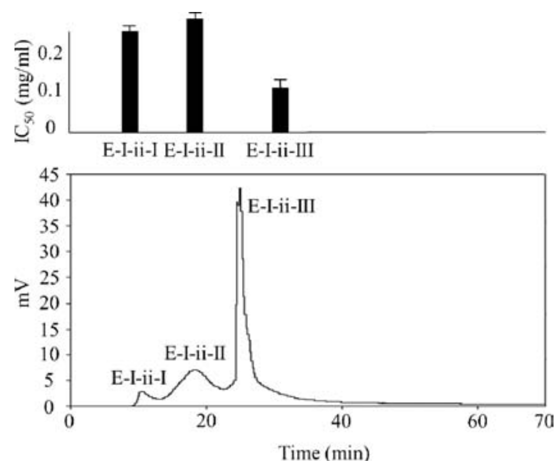

Figure 4. Reverse-phase HPLC trace on a GPC SB-802.5 column (300 × 8.0 mm) of active fraction E-I-ii-III eluted on the HPLC (lower panel) and hydroxyl radical scavenging activities (upper panel) of the fractions. HPLC operation was carried out at a flow rate of 0.5 mL/min using a UV detector at 215 nm.

collected, fraction E-I-ii-III exhibited the strongest ACE inhibitory activity. E-I-ii-III was rechromatographed by a fourth RP-HPLC step on a GPC SB-803 column (300 × 8.0 mm). After this fourth purification step, a purified antihypertensive peptide was finally obtained (Figure 5). The amino acid sequence of the purified peptide, which had a molecular mass of 693.90 Da, was measured by ESI-QTOF (Shinhan Scientific, Ochang, Korea) and shown to be Trp-Tyr-Pro-Ala-Ala-Pro (Figure 6). The purified peptide exhibited the highest antihypertensive activity with an IC_{50} value of 137 μM. Biological activities of protein hydrolysates are related to the amino acid composition, sequence, size, and configuration of peptides. With regard to the ACE-inhibitory activity specifically, binding to ACE is strongly influenced by the C-terminal tripeptide sequence. Although the structure−activity relationship of food-derived ACE inhibitory peptides has not yet been fully established, ACE prefers inhibitors containing hydro-

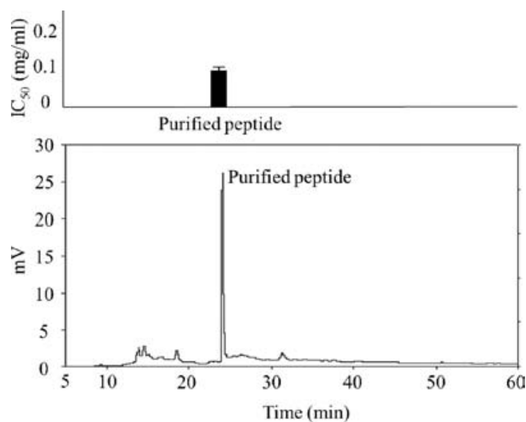

Figure 5. Active fraction with the highest antihypertensive activity finally purified on a GPC SB-803 column (300 × 8.0 mm) using the purified peptide eluted on the HPLC (lower panel) and ACE inhibitory activities (upper panel) of the fractions. HPLC operation was carried out at a flow rate of 0.1 mL/min using a UV detector at 215 nm.

phobic amino acid residues at each of the three C-terminal positions.[26] Significant ACE inhibitory peptides isolated from porcine skeletal muscle protein hydrolysates included Met-Asn-Pro, Thr-Asn-Pro, Asn-Pro-Pro, and Ile-Thr-Thr-Asn-Pro.[27] In addition, an ACE inhibitory peptide was previously isolated and purified from the hydrolysates of duck meat protein. The amino acid sequence of this purified inhibitory peptide was Glu-Asp-Leu-Glu.[28] Most peptides that inhibit ACE have at least one proline residue,[29,30] which was also the case for the peptide isolated in this study.

Our results showed that the purified peptide efficiently quenched ACE sources. The ACE inhibitory activity of the purified peptide was similar to that of captopril (data not shown).

The inhibition mechanism of the purified ACE inhibitory peptide from duck skin byproducts was determined to be competitive using Lineweaver−Burk plots (Figure 7). Thus, the ACE inhibitor from duck skin byproducts binds competitively with the substrate at the active site of ACE. An ACE inhibitory fraction isolated from fermented oyster sauce also functioned through a competitive mechanism.[31]

The systolic blood pressure of Wistar rats was approximately 120 mmHg (110−131 mmHg) during the duration of the study (Figure 8). In SHR, after injection of the ACE inhibitor (synthetic peptide), the mean systolic blood pressure decreased to 160 mmHg after 0.5 h and continued to decrease up to 6 h after injection. Injection of the peptide was shown to lower the blood pressure more than injection of captopril, and this

y ions : 860.39 674.31 511.25 414.20 343.16 272.12

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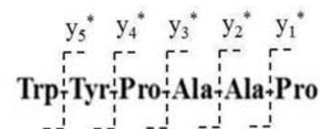

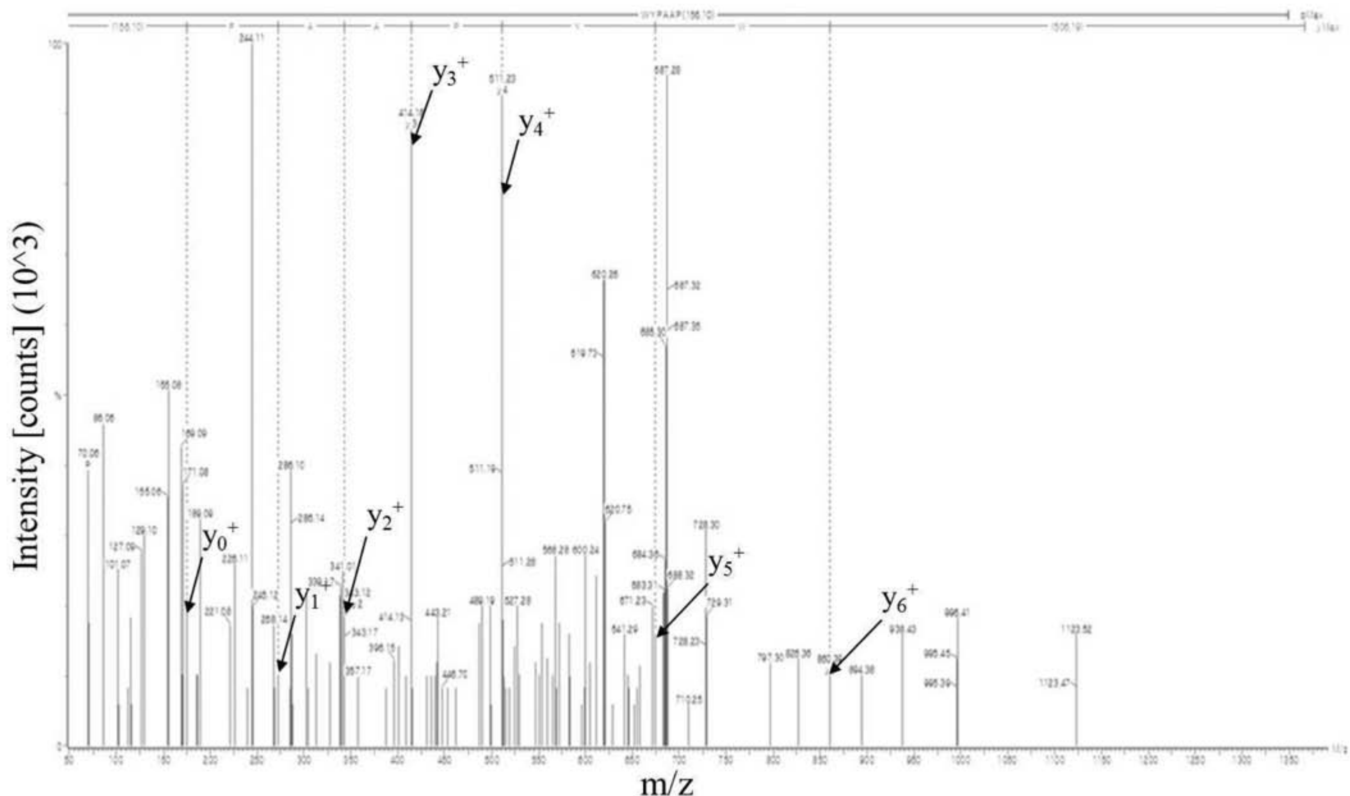

Figure 6. Identification of molecular mass and amino acid sequence of purified peptide. Sequencing of the active peptide was acquired over the m/z range 50−2500 and sequenced using the PepSeq de novo sequencing algorithm.

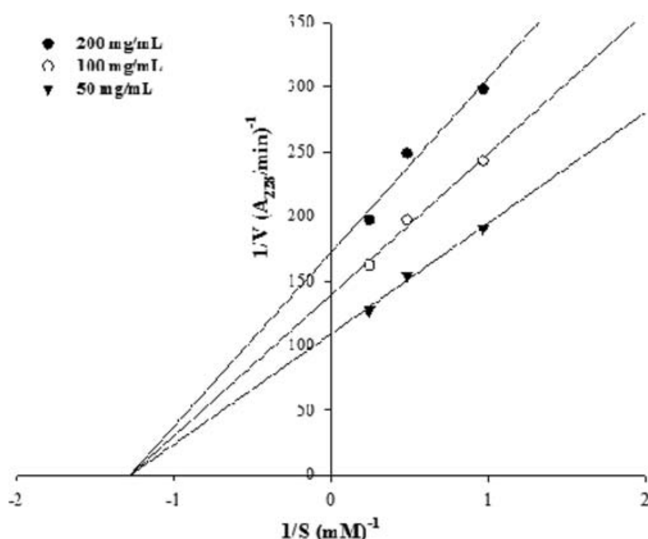

Figure 7. Lineweaver−Burk plots of ACE inhibitory activity in the presence of the inhibitor: ●, 200 mg/mL; ○, 100 mg/mL inhibitor; ▼, 50 mg/mL inhibitor.

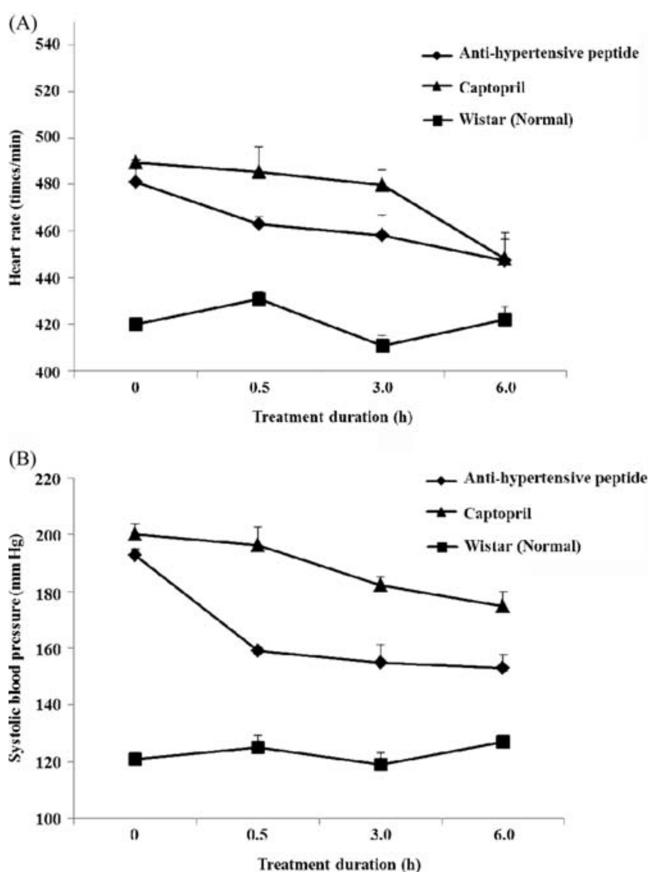

Figure 8. Change in heart rate (A) and systolic blood pressure (B) of SHR after administration of the ACE inhibitor. Intravenous injection of captopril and synthesis peptide was performed at doses of 10 mg/kg and 1.0 mg/kg body weight, respectively, and the heart rate and systolic blood pressure were measured 0, 0.5, 3, and 6 h after administration. Points on the figure represent mean values ± SEM of seven rats.

difference was statistically significant at 6 h, when systolic blood pressure was lowered by 19% (P = 0.037, one-way ANOVA).

The antihypertensive activity of the ACE inhibitor was evaluated by measuring changes in the heart rate and systolic blood pressure at 0.5, 3, and 6 h after intravenous injection of 1.0 mg/kg of body weight. There was no change in heart rate and systolic blood pressure in the Wistar rats during the investigation period. As shown in Figure 8, a decrease in heart rate and systolic blood pressure was observed after 0.5 h. The ACE inhibitor significantly lowered the heart rate and systolic blood pressure 6 h after injection, and this decrease was greater than that achieved using captopril. Many ACE inhibitory peptides have recently been separated from natural products or food proteins, and their antihypertensive effects have been evaluated in animal models (SHR). The purification and characterization of ACE inhibitory peptides were previously reported from Alaskan pollock (*Theragra chalcogramma*) skin. The isolated peptides were composed of Gly-Pro-Leu and Gly-Pro-Met.[32] Another study also reported the purification and characterization of an ACE inhibitory peptide from the rotifer *Brachionus rotundiformis*, with an amino acid sequence of Asp-Asp-Thr-Gly-His-Asp-Phe-Glu-Asp-Thr-Gly-Glu-Ala-Met, which had a molecular mass of 1538 Da.[33]

The results of this study suggest that an antihypertensive peptide derived from duck skin byproducts could be used to develop novel pharmaceutical agents.

AUTHOR INFORMATION

Corresponding Author

*Phone: +82-43-840-3588. Fax: +82-43-852-3616. E-mail: parkpj@kku.ac.kr.

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Notes

The authors declare no competing financial interest.

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