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Action Mechanism of an Angiotensin I-Converting Enzyme Inhibitory Peptide Derived from Chicken Breast Muscle

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In a previous study, we isolated the inhibitory peptide (P4 peptide, Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe) for angiotensin I-converting enzyme (ACE) from chicken breast muscle extract possessing hypotensive activity for spontaneously hypertensive rats (SHRs). This study was performed to elucidate the peptide's action mechanisms of inhibiting ACE. Intravenous administration of synthetic P4 peptide resulted in significant drops in the blood pressures of SHRs. As Dixon plots indicate, the P4 peptide showed high affinity toward ACE ($K_i = 11.48$ μM) and only 10% of the total amount of the P4 peptide was decomposed. The analyses of the relationship between the ACE inhibitory activity and structure of the P4 peptide clarified that Hyp-Gly-Leu-Hyp-Gly-Phe showed a stronger activity ($IC_{50} = 10$ μM) than the P4 peptide ($IC_{50} = 46$ μM). When Phe at the C-terminus of the P4 peptide was deleted, IC_{50} changed to 25000 μM, indicating that Phe at the C-terminus of the peptide is very important for ACE inhibitory activity.

KEYWORDS: Angiotensin I-converting enzyme inhibitor; spontaneously hypertensive rats; hypotensive activity; chicken breast muscle extract; peptide

INTRODUCTION

Lifestyle-related disease has increased every year. Hypertension is one such disease, and has raised the risks of stroke and cerebral infarction (*1*, *2*). Blood pressure is controlled by various regulatory factors in the body. Angiotensin I-converting enzyme (ACE) is one such factor: ACE converts angiotensin I, which is produced from angiotensinogen, into angiotensin II, a pressor hormone. Angiotensin II causes peripheral vasoconstriction, reabsorption of Na in the kidneys, and increases in the amounts of body fluids (*3*). Angiotensin III, which is produced by decomposing angiotensin II, controls the production of aldosterone (*4*) and raises the blood pressure. Thus, inhibition of ACE results in inhibition of the conversion of angiotensin I into angiotensin II and is one of the most effective methods for suppressing rises in blood pressure.

A number of food components have been found to have ACE inhibition activities. ACE inhibitory peptides have been found in and isolated from hydrolyzates of gelatin (*5*), milk casein (*6*−*8*), endoderm proteins (*9*−*11*), and fish proteins (*12*−*14*). Most of the peptides have aromatic amino acids, such as Pro, Tyr, or Trp, at their C-termini. Cheung et al. (*15*) also reported that the ACE inhibitory activity is stronger when there is a dipeptide, such as -His-Leu, -Phe-Arg, and -Ala-Pro, at the C-terminus. ACE belongs to the family of metalloproteases including thermolysin and carboxypeptidase A. It is thought that one key active site residue in ACE is the H*EXX*H motif involved in the zinc-ligating residues in the human ACE C-domain. Furthermore, the positively charged groups at both active sites form ionic bonds with the negatively charged terminal carboxyl groups of the substrates (*16*, *17*). However, the substrate binding site residues are still ambiguous, and the catalytic mechanism in ACE is not well clarified.

We discovered that administration of a hydrothermal extract of chicken breast muscle (chicken breast muscle was boiled in aqueous solution, pH 4.5, and then hydrolyzed by *Aspergillus* protease) prevented rises in blood pressure in spontaneously hypertensive rats (SHRs) by 50 mmHg compared with the case in which saline was administered, as described in our previous paper (*18*). We also found an ACE inhibitory peptide [Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe (P4 peptide)] in the extract digested by gastric enzymes (trypsin−chymotrypsin and small intestinal enzymes). The peptide showed an IC_{50} of 42 μM and was as active as a lactotripeptide and a sardine peptide, which have been reported to be ACE inhibitory peptides. Thus, the peptide is a promising ingredient for preparing functional foods, and its characteristics should be analyzed in detail.

In this study, we synthesized the P4 peptide, examined whether the synthesized peptide suppressed rises in blood pressure, and clarified the inhibition mechanisms and active regions of the P4 peptide.

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MATERIALS AND METHODS

Materials. ACE from rabbit lung, trypsin, and chymotrypsin were purchased from Sigma Chemical Co. (St. Louis, MO). Amino acid derivatives for peptide syntheses were purchased from Shimadzu Co. (Kyoto, Japan). Hippuryl-L-histidyl-L-leucine (Hip-HL) was obtained from Peptide Institute Inc. (Osaka, Japan). Other chemical reagents were of reagent grade or better.

Measurement of Blood Pressure and Heartbeat in SHRs. Eight-week-old male SHRs were fed a commercial nonpurified diet (AIN-76; Oriental Yeast, Tokyo, Japan) and water for 2 weeks ad libitum in an environment-controlled room (23 °C, 55% humidity), and then either only saline or saline containing a peptide (30 mg/kg of mass) was administered through a tail vein. Their tail systolic blood pressure and heartbeat were determined by the tail-cuff method using a plethysmographic tail apparatus (Softron 98A; Softron Co., Tokyo, Japan). Student's *t* test was used to analyze whether there were significant differences among the data.

Assaying of Inhibitory Activity toward ACE. The inhibitory activity of peptides toward ACE was assayed according to the method reported by Cheung (*15*). The following assay components, in a final volume of 0.25 mL, were incubated at 37 °C for 30 min: 100 mM sodium borate buffer (pH 8.3), 5 mM Hip-HL, 500 mM NaCl, 20 mU of rabbit lung ACE, and peptides. The enzyme reaction was stopped by the addition of a 1 N HCl solution. The rate of hydrolysis of Hip-HL was determined by measuring the absorbance of the released hippuric acid at 228 nm after successive extraction with ethyl acetate, evaporation of the ethyl acetate, and dissolution of the residue in water. The ACE inhibitor concentration required to inhibit 50% of the ACE activity under the conditions described above was expressed as IC_{50}, which was calculated using the net weight of the protein hydrolysate.

Analysis of the N-Terminal Amino Acid Sequences of Peptides. The N-terminal amino acid sequences of the isolated hypotensive peptides were determined with a protein sequencer, G1005A (Hewlett-Packard Co., Wilmington, DE).

Peptide Syntheses. Peptides were synthesized by the fluorenylmethoxycarbonyl (Fmoc) strategy using a simultaneous multiple-peptide synthesizer (model PSSM-8; Shimadzu, Kyoto, Japan) according to the method reported by Nokihara et al. (*19*). After being synthesized, a peptide was purified by HPLC on an ODS column (PEGASIL-300, 20 × 250 mm; Senshu, Tokyo, Japan) with a linear gradient of 0−50% CH_3CN containing 0.1% trifluoroacetic acid in 100 min (flow rate, 5.0 mL/ min; monitoring, 220 nm). Furthermore, the molecular mass of an isolated peptide was determined by mass spectrometry with an ESI mass spectrometer, LC-Q (Thermo Finnigan, San Jose, CA).

Determination of the ACE Inhibition Manner. The K_i value was obtained from Dixon plots using three concentrations of the P4 peptide (0.05−1 mM) and two concentrations of the substrate Hip-HL (1.25−2.5 mM). $1/v$ is a reciprocal number of absorbance at 228 nm. The ACE inhibition manner of the synthetic peptide was investigated according to the method of Fujita et al. (*20*). A 6 mM concentration of the peptide was incubated with 10 mU of ACE at 37 °C for 3 h. After the incubation, the mixture was heated to 100 °C for 10 min to stop the reaction of the ACE. The supernatant from centrifugation (10000*g*, 10 min) was applied on an ODS, column and the chromatographs before and after incubation with ACE were compared. The peaks were recovered, and the molecular weights were determined by electrospray ionization mass spectrometry.

Statistical Evaluations. Data on blood pressure were evaluated by one-way ANOVA, followed by Student's *t* test to compare the mean of each dose group with that of the control group. The probability level used to determine statistical significance was $P < 0.05$.

RESULTS

Changes in Blood Pressure by Administration of the P4 Peptide. Changes in blood pressure when the P4 peptide was injected through a vein are shown in **Figure 1**. The blood pressure of the group to which the P4 peptide was administered was significantly lower than that of the control group, and the effect continued for over 30 min. These blood pressure levels were returned to the base pressure after 60 min (control, 152 ± 3 mmHg; P4 peptide, 156 ± 5 mmHg).

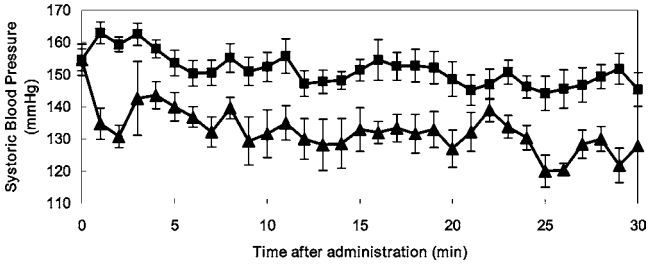

Figure 1. Changes in blood pressure on intravenous administration of the P4 peptide: ■, synthetic P4; ---, saline (control). The blood pressure of 8-week-old SHRs was measured by the tail-cuff method after intravenous administration of the P4 peptide (30 mg/kg of rat mass). Average blood pressure values in five SHRs are shown. Each bar shows the standard error. The values during the whole time of 30 min except for at 4 and 22 min were significantly lower than those of the control ($P < 0.05$).

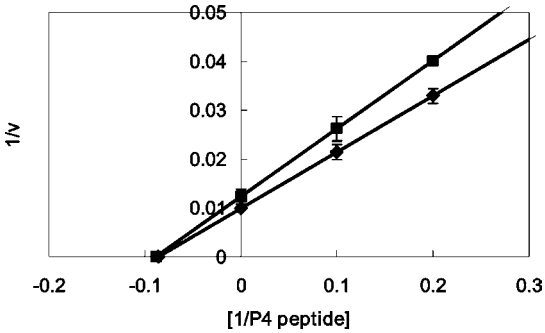

Figure 2. Manner of ACE inhibition by the P4 peptide and inhibition constant: ◆, 2.5 mM substrate; ■, 1.25 mM substrate. Each bar shows the standard error. Values are the means of triplicate analyses. From the intersecting point ($1/K_i = 0.087$ mM^{-1}), $K_i = 11.48$ μM.

Manner of Inhibition by the P4 Peptide. The inhibitory mechanism by the P4 peptide was analyzed in detail by determining the inhibitory constant (K_i). The inhibitory activity was measured by changing the concentration of the P4 peptide at a constant concentration (1.25 and 2.5 mM) of the substrate Hip-HL. The results were plotted (Dixon plot) to determine the mode of inhibition and K_i (**Figure 2**). Because the plots obtained by changing the substrate concentration intersect with the *x* axis, the inhibition of ACE by the peptide seemed to be noncompetitive inhibition. From the value at which the line intersects with the *x* axis, the K_i value was calculated to be 11.48 μM.

We then investigated whether the P4 peptide is decomposed when the peptide is incubated with ACE, by using HPLC. The resulting HPLC patterns are shown in **Figure 3**. Incubation with ACE resulted in two additional peaks (peak 1 and peak 2) besides the peak for the original peptide (P4 peptide). The area of the HPLC plot showed that about 90% of the P4 peptide remained. The molecular weights of two additional peaks were measured by using the mass spectrometer. We could not determine the molecular weight of peak 1, and thus, its structure is unknown. On the other hand, the molecular weight corresponding to peak 2 was 804, which is the same as that of a peptide lacking Gly-Phe at the C-terminus of the P4 peptide.

Furthermore, the inhibitory activity of the peptide solution after incubation with ACE was also investigated. The IC_{50} of the peptide solution was changed to 52.5 μM (the IC_{50} before incubation was 42 μM).

Relationship between the Inhibitory Activity and Structure of the P4 Peptide. The relationship between the inhibitory

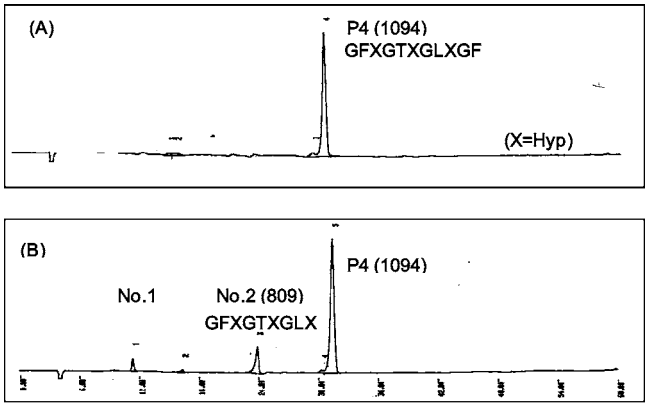

Figure 3. Stability of the P4 peptide in the presence of ACE. The P4 peptide was applied on an ODS column by HPLC (**A**). The P4 peptide treated with ACE at 37 °C for 30 min was applied on the same column (**B**).

Table 1. Inhibitory Activity of the Peptides Related to the P4 Peptide

	Synthetic peptide	IC_{50}(μM)
P4	Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe	26
No.1	Hyp-Gly-Phe	433
No.2	Hyp-Gly-Leu-Hyp-Gly-Phe	10
No.3	Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe	19
No.4	Phe-Hyp-Gly	171
No.5	Phe-Hyp-Gly-Thr-Hyp-Gly	406
No.6	Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly	25000

activity and structure of the P4 peptide was investigated by synthesizing the peptides related to the P4 peptide and measuring their ACE inhibition (**Table 1**). A peptide consisting of three residues at the C-terminus showed an inhibitory activity that was 1/20th of that of the original peptide (IC_{50} = 443 μM). A peptide that consisted of three residues at the N-terminus also showed an inhibitory activity that was 1/10th of that of the original peptide (IC_{50} = 171 μM). On the other hand, a peptide consisting of six amino acids at the C-terminus showed a higher activity than the original peptide. Removal of the C-terminus produced marked drops in activity, and the drop was especially large when Phe was removed from the C-terminus.

DISCUSSION

Intravenous administration of the P4 peptide caused rapid drops in the blood pressures of SHRs. The mean value of the drop was 20 mmHg. From this result, the P4 peptide was confirmed to have hypotensive activity toward SHRs. Some ACE inhibitory peptides derived from food proteins have been shown to possess hypotensive activity toward SHRs. Intraperitoneal administration of Leu-Arg-Pro of α-zein origin at 30 mg/kg of body mass has been reported to reduce the blood pressures of SHRs by about 15 mmHg (*11*). Intravenous administration of Ile-Lys-Pro of dried bonito origin together with ACE suppressed rises in the blood pressures of Wister rats (*13*). In our previous paper, we showed that oral administration of chicken breast muscle thermal extract reduced blood pressure by up to 50 mmHg in 3 h (*1*). In this paper, the intravenous administration of the P4 peptide derived from chicken breast muscle was clarified to cause the drop in blood pressure immediately after the administration. Since the P4 peptide was isolated from the hydrolysate of chicken breast muscle treated with digestive enzymes, this peptide seemed to be produced by digestive enzymes within the body and to drop the blood pressures of SHRs.

The manner of ACE inhibition of the P4 peptide was analyzed (**Figure 2**). The Dixon plot showed noncompetitive inhibition. The K_i value calculated from the data in **Figure 2** was 11.5 μM, which is much lower than the value of Hip-HL (2.3 mM), indicating that the affinity of the P4 peptide for ACE is higher than those of ACE substrates and the inhibition manner is noncompetitive. However, the result of HPLC profiles (**Figure 3**) showed that the P4 peptide was partially hydrolyzed by ACE. After incubation of the P4 peptide with ACE for 3 h, approximately 10% of the peptide was decomposed. This hydrolysis seems to cause the change of IC_{50} from 42 to 52.5 μM. Thus, the long-time incubation of the P4 peptide with ACE was also clarified to cause hydrolysis of the P4 peptide. The peptide Gly-Phe released from the P4 peptide by long-time incubation is reported to have weak inhibitory activity (IC_{50} = 630 μM) (*15*). It appears that decomposition of the P4 peptide directly affects its inhibitory activity.

As described in the Introduction, Cheung et al. reported that aromatic amino acids at the C-terminus of the peptides are important for inhibition of ACE (*15*). The ACE inhibitory peptides, such as Phe-Phe-Val-Ara-Pro (*6*) and Ile-Pro-Pro (*8*) in hydrolysates of casein, Val-His-Leu-Pro-Pro (*9*) and Leu-Gln-Pro (*10*) in hydrolysates of corn proteins, and Phe-Gln-Pro, Ile-Tyr, Asp-Tyr-Gly-Leu-Tyr-Pro, and Ile-Lys-Pro-Leu-Asn-Tyr (*12*) in hydrolysates of sardine proteins, have an amino acid with a ring structure at their C-termini. Met-Phe (*21*), Thr-Phe (*22*), Val-Phe (*15*), Phe-Asn-Phe and Leu-Thr-Phe (*23*), and Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (*24*) also have been reported as ACE inhibitory peptides which contain Phe at their C-termini. In this study, when peptides related to the P4 peptides were synthesized and their ACE inhibition was measured, the activity of Hyp-Gly-Phe corresponding to the C-terminus was reduced to 1/20th of that of the original P4 peptide (**Table 1**). On the other hand, the activities of Hyp-Gly-Leu-Hyp-Gly-Phe and Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe were almost the same as that of the original P4 peptide, suggesting that Hyp-Gly-Leu at the fourth to sixth positions in the C-terminus is responsible for the affinity with the enzyme. The IC_{50} of Phe-Hyp-Gly on the N-terminus was only 171 μM, and its ACE inhibitory activity was 1/10th of that of the original P4 peptide. The presence of an antepenultimate aromatic amino acid such as Pro, Hyp, Trp, Tyr, and Phe in a substrate or competitive inhibitor has been shown to enhance binding of peptide to ACE (*15*). This rule seems to apply to the inhibition of all peptides except for peptide no. 1. Although the structure of peptide no. 6 was similar to that of peptide no. 3, peptide no. 6 showed a very small activity. The difference in the structure of both peptides is the position of Phe. This result confirmed that Phe at the C-terminus is very important for ACE to possess inhibitory activity.

In conclusion, (i) the synthesized P4 peptide derived from chicken skeletal muscle possessed hypotensive activity toward SHRs, (ii) the P4 peptide showed strong affinity toward ACE and inhibited ACE in a noncompetitive manner, and (iii) the presence of an aromatic amino acid at the antepenultimate position and Phe at the C-terminus in the P4 peptide seemed to play an important role in its inhibitory activity.

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