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Angiotensin I-Converting Enzyme Inhibitory Peptides in a Hydrolyzed Chicken Breast Muscle Extract

AI SAIGA,[†] TOMOYUKI OKUMURA,[‡] TOSHIKAZU MAKIHARA,[‡] SHINICHIRO KATSUTA,[§] TSUYOSHI SHIMIZU,[§] RYOJI YAMADA,[‡] AND TOSHIHIDE NISHIMURA*[,†]

Graduate School of Biosphere, Hiroshima University, Higashi-hiroshima 739-8528, Japan; Japan Research and Development Center, Nippon Meat Packers, Inc., Tsukuba, Ibaraki 300-2646, Japan; and School of Medicine, Fukushima Medical University, Fukushima 960-1295, Japan

The blood pressure of spontaneously hypertensive rats (SHRs) decreased after oral administration of an extract prepared from chicken breast muscle, falling maximally to 50 mmHg lower than before. This effect continued for at least 4 h after administration. The peptides possessing hypotensive activity in the chicken extract were examined by measuring the inhibitory activity (IC_{50}) against angiotensin I-converting enzyme (ACE). The inhibitory activity of the chicken extract was 1060 mg%, whereas the activity of the extract treated with an *Aspergillus* protease and gastric proteases (trypsin, chymotrypsin, and intestinal juice) became stronger, reaching 1.1 mg%. Peptides in this hydrolysate of the extract were isolated by HPLC on a reversed-phase column, and their N-terminal sequences were analyzed. Three peptides possessed a common sequence, Gly-X-X-Gly-X-X-Gly-X-X, which was homologous with that of collagen. The peptide Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe showed the strongest inhibitory activity (IC_{50} = 42 μM).

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

INTRODUCTION

It is well-known that chicken soup is an extremely nutritious and palatable food, because it contains a lot of nitrogen compounds including free amino acids and possesses a stronger umami and brothy taste than beef and pork soups. Furthermore, it is thought in China that chicken soup has physical functions such as improvement of the blood circulation or a weak constitution (*1*).

Recently, peptides derived from proteins in foods have been shown to regulate physical functions in the alimentary, neural, and circulatory systems. For example, gluten exorphin isolated from wheat gluten hydrolysate releases insulin like an opioid hormone (*2*, *3*). β-Casomorphin derived from casein has been shown to regulate peristalsis of the small intestine (*4*). Peptides derived from soybean and pork proteins can suppress the increase in cholesterol in the serum after a meal (*5*, *6*). A peptide in a casein hydrolysate treated with pepsin promotes calcium absorption, being utilized as a functional material (*7*). Furthermore, peptides possessing hypotensive activity have been discovered in hydrolysates of gelatin (*8*), casein (*9*−*14*), zein (a maize endosperm protein) (*15*−*17*), and fish muscle proteins (*18*−*21*).

Among many functional peptides, ones possessing hypotensive activity are thought to be useful as functional food materials for high blood pressure patients. This activity is mainly due to the inhibition of the angiotensin I-converting enzyme (ACE) (EC 3.4.15.1), which plays an important role in the regulation of blood pressure in two ways. One is the conversion of the inactive decapeptide angiotensin I into a strong vasoconstrictor and salt-retaining octapeptide, angiotensin II. The other is the inactivation of the vasodilator and natriuretic nanopeptide bradykinin. Cheung et al. (*22*) systematically synthesized many peptides and showed that the carboxyl-terminal sequences (−HL, −FR, and −AP) of their peptides are involved in the inhibition of ACE. A tripeptide, Leu-Arg-Pro, in a zein hydrolysate has also been reported to exhibit hypotensive activity in spontaneously hypertensive rats (SHR), decreasing their blood pressure by 15 mmHg after a 30 mg/kg intravenous injection (*17*). An octapeptide, Pro-Thr-His-Ile-Lys-Trp-Gly-Asp, from a tuna muscle hydrolysate has been shown to exhibit inhibitory activity toward ACE and hypotensive activity in SHR (*18*). A lacto-tripeptide, Val-Pro-Pro, from casein has hypotensive activity and is utilized for functional foods as an antihypertensive agent (*23*). Although many peptides possessing hypotensive or

* Author to whom correspondence should be addressed (telephone +81-824-24-7984; fax +81-824-24-7984; e-mail toshixy@hiroshima-u.ac.jp).
[†] Hiroshima University.
[‡] Nippon Meat Packers Inc.
[§] Fukushima Medical University.

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ACE inhibitory activity have been discovered in hydrolysates of food proteins, as described above, there is little information on the functions of ACE inhibitory peptides derived from animal meat proteins.

In the present study, we investigated the hypotensive effect of a chicken muscle extract in SHR. ACE inhibitory peptides were isolated from the hydrolyzed chicken extract, and their structures were clarified. Furthermore, the peptide exhibiting the highest inhibitory activity was synthesized and characterized.

MATERIALS AND METHODS

Materials. Chicken breast muscle and fresh porcine small intestines were obtained from Nippon Meat Packers Inc. (Osaka, Japan). *Aspergillus* protease was bought from Sankyo Co. (Tokyo, Japan). 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.

Preparation of an Extract of Chicken Breast Muscle. A chicken extract was made from chicken breast muscle according to the following method. One kilogram of chicken breast muscle blocks was boiled in a 1.5 L aqueous solution (pH 4−4.5) for 3.5 h to extract proteins from muscle blocks adequately. After boiling, the extract was filtered and centrifuged to remove the precipitate. Furthermore, the supernatant was concentrated with Brix 25 and used as the chicken extract.

Preparation of Small Intestine Juice. Immediately after slaughter, fresh porcine small intestines were removed from the carcasses and then washed with cold 0.85% NaCl. Small intestinal epithelia and mucosae were collected using cover glasses and then homogenized with 7 volumes (w/v) of 0.1 M Tris-HCl buffer (pH 7.2) containing 12% glycerol (1000 rpm, 3 min). After centrifugation (30000*g*), the supernatant was filtered and dialyzed against 10 mM Tris-HCl buffer (pH 7.2). Protein concentrations were determined with a protein assay kit (Bio-Rad, Hercules, CA) according to the colorimetric method.

Digestion of the Chicken Extract with Proteases. The chicken extract was first hydrolyzed with 0.06% *Aspergillus* protease at 50 °C and pH 7.0 for 1 h. After this reaction had been stopped by boiling for 10 min, the supernatant was further hydrolyzed by successive treatment with 1% trypsin/chymotrypsin and small intestinal juice at 37 °C and pH 7.0 for 1 h and 30 min, respectively. Each digestion was also stopped by boiling for 10 min to inactivate proteases. After treatment, all hydrolysates were centrifuged to remove the precipitates.

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 saline or a protein hydrolysate (1 g/kg weight) dissolved in saline was administered orally. Their tail systolic blood pressure and heartbeat were determined at 0, 1, 2, 3, and 4 h after the oral administration by tail-cuff method using a plethysmographic tail apparatus (Softron 98A; Softron Co., Tokyo, Japan).

Assay of Inhibitory Activity toward ACE. The inhibitory activity of a chicken extract or peptides toward ACE was assayed according to the method reported by Cheung (*22*). 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 milliunits of rabbit lung ACE, and a chicken extract or 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.

Separation and Purification of Hypotensive Peptides from a Hydrolyzed Chicken Extract. Peptides in a chicken extract digested with trypsin, chymotrypsin, and small intestine juice were first divided into two groups, with molecular masses of less and more than 1000 Da, respectively, using an ultrafiltration membrane (Millipore Co., Bedford, MA). Peptides in the former group possessing high ACE inhibitory activity were purified by HPLC on an ODS column (22 × 250 mm and 4.6 × 250 mm; Senshu, Tokyo, Japan) using a linear gradient of CH_3CN (8−40% in 80 min or 8−40% in 64 min, respectively) containing 0.1% trifluoroacetic acid at the flow rate of 1.0 mL/min. The elution peaks were monitored at 220 nm.

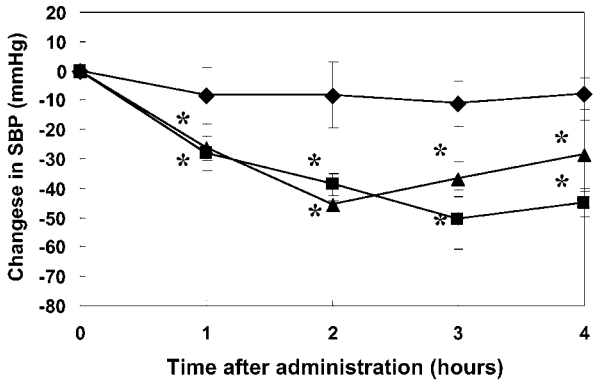

Figure 1. Effect of chicken extract administration on the blood pressure of spontaneously hypertensive rats: ◆, control (saline); ▲, protease-treated extract; ■, untreated extract. The blood pressure of 8-week-old SHRs was measured by the tail-cuff method after oral administration of samples (1.0 g/kg of rat weight). Average blood pressure values in five SHRs are shown. Each bar shows the standard error. *, significantly different from the control (*, $p < 0.05$) with Dunnett's test.

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. (*24*). 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 at 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).

Statistical Evaluations. Data on blood pressure and heartbeat count were evaluated by one-way ANOVA, followed by Dunnett's 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

Hypotensive Activity of Protein Hydrolysates in SHR. The blood pressure of SHRs was measured at 0, 1, 2, 3, and 4 h after oral administration of the *Aspergillus* protease-treated or untreated chicken extract. The administration of a treated or an untreated chicken extract significantly lowered the blood pressure of SHRs 1 h later, whereas no decrease in blood pressure was observed in control SHRs fed no chicken extract. This effect of both chicken extracts on blood pressure continued for at least 4 h. The SHRs fed the untreated chicken extract exhibited the greatest decrease, 50 mmHg, at 3 h after administration. The administration of the chicken extract treated with the *Aspergillus* protease also caused a greatest decrease, 45 mmHg, at 2 h after administration (**Figure 1**). There is a little difference in continuity between the *Aspergillus* protease-treated and untreated chicken extracts. This difference may be due to the difference in the speeds of hydrolysis of the two chicken extracts by gastric proteases in SHRs.

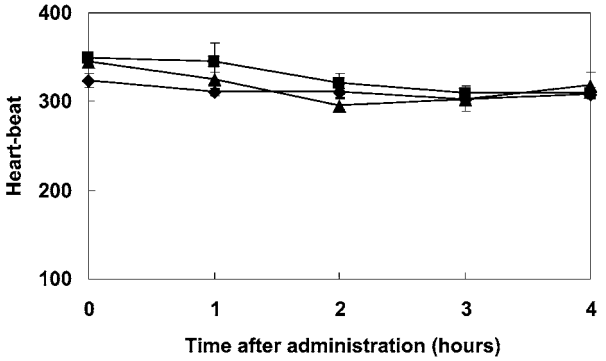

Figure 2. Effect of chicken extract administration on the heartbeat of spontaneously hypertensive rats: ◆, control (saline); ▲, protease-treated extract; ■, untreated extract. The heartbeat of 8-week-old SHRs was measured by the tail-cuff method after oral administration of sample (1.0 g/kg of rat weight). Average heartbeat counts in five SHRs are shown. Each bar shows the standard error.

Table 1. Inhibitory Activity of Each Chicken Extract against Angiotensin I-Converting Enzyme

	untreated chicken extract	protease-treated[b]	ultrafiltration after protease treament[b]		lacto-tripeptide V-P-P
			MW < 1000	MW > 1000	
IC_{50}[a] (mg%)	1060 ± 13.0	1.1 ± 0.4	0.8 ± 0.2	14 ± 2.5	13

[a] Concentration of peptide needed to inhibit 50% of the ACE activity. [b] Chicken extract treated with *Aspergillus* protease and gastric enzymes.

The heartbeat in SHRs was also measured after administration in order to check the effect of the administration of chicken extracts on the physical condition. As shown in **Figure 2**, there was no change in the heartbeat of SHRs at 1, 2, 3, and 4 h after oral administration, suggesting that the administration of chicken extracts did not have a bad effect on the circulatory system of SHRs.

Inhibition of ACE Activity by Chicken Extract Hydrolysates. Some peptides in protein hydrolysates have been shown to suppress blood pressure in SHRs through the inhibition of ACE. To examine the peptides in chicken extracts involved in the lowering of the blood pressure, ACE inhibitory activities of chicken extracts were first examined. Because peptides in chicken extracts were further hydrolyzed by gastric systems after administration, the ACE inhibitory activity of the chicken extracts treated with trypsin, chymotrypsin, and small intestine juice was also measured.

The untreated chicken extract exhibited very low inhibitory activity (IC_{50} = 1060 mg%). This extract treated with an *Aspergillus* protease, trypsin/chymotrypsin, and small intestine juice, however, exhibited high inhibitory activity (IC_{50} = 1.1 mg%). Small peptides (<1000 Da) in this extract separated with an ultrafilter membrane showed higher activity (IC_{50} = 0.8 mg%) than those with molecular masses of >1000 Da (**Table 1**). These results suggested that proteins and peptides would exhibit higher inhibitory activity on hydrolysis by gastric proteases.

Purification of ACE Inhibitory Peptides. The ACE inhibitory peptides in a chicken extract treated with the *Aspergillus* protease and gastric proteases were separated by HPLC on a reversed-phase column (**Figure 3A**). The separated peptides were divided into seven groups (F0−F6) according to the retention time. The ACE inhibitory activity of each group was measured. Among all groups, fraction F6 showed the highest

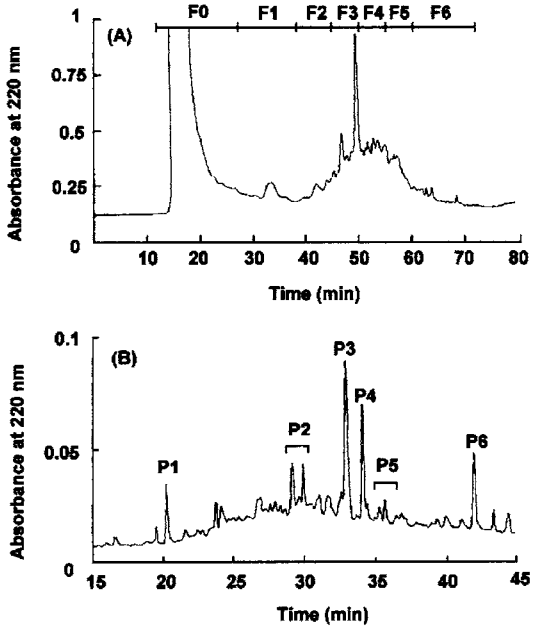

Figure 3. Purification of ACE inhibitory peptides obtained from chicken extract by HPLC on an ODS column: (A) first HPLC; (B) second HPLC. F6 on the first HPLC was applied to the second HPLC. Gradient conditions for elution on both HPLCs are given under Materials and Methods.

Table 2. Angiotensin I-Converting Enzyme Inhibitory Activity of the Peptide Fractions Obtained on First HPLC

fraction	IC_{50} (mg%)	fraction	IC_{50} (mg%)
F0	1.417 ± 0.667	F4	0.173 ± 0.015
F1	0.374 ± 0.042	F5	0.110 ± 0.016
F2	0.176 ± 0.027	F6	0.084 ± 0.011
F3	0.159 ± 0.016		

[a] Fraction numbers correspond to F0–F6 in **Figure 3A**.

Table 3. Angiotensin I-converting Enzyme Inhibitory Activity of the Peptide Peaks Obtained on Second HPLC

peak	IC_{50} (mg%)	peak	IC_{50} (mg%)
P1	0.204 ± 0.016	P4	0.068 ± 0.003
P2	0.169 ± 0.019	P5	0.192 ± 0.017
P3	0.070 ± 0.004	P6	0.081 ± 0.003

[a] Peak numbers correspond to P1–P6 in **Figure 3B**.

inhibitory activity and fraction F5 exhibited the second highest (**Table 2**). Therefore, peptides in F6 were further separated by HPLC on the same column (**Figure 3B**). The ACE inhibitory activities of the six isolated peptides (P1−P6) were measured. Three peptides, designated P3, P4, and P6, exhibited strong inhibitory activities against ACE. On the other hand, the inhibitory activities of P1, P2, and P5 were weak (**Table 3**).

Analyses of the Structures of ACE Inhibitory Peptides. The structures of the six peptides isolated by HPLC on an ODS column were analyzed with a protein sequencer. The sequences of four peptides were clarified, as shown in **Table 4**. Among them, P3, P4, and P6 possessed a unique and repeated sequence, Gly-X-X-Gly-X-X-Gly-X-X-Gly (X, amino acid). Homology analysis with known proteins showed that these peptides were derived from collagen.

ACE Inhibitory Activity of Synthesized Peptides. To confirm the ACE inhibitory activity of the isolated peptide (P4), Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe was synthesized with a peptide synthesizer, and its inhibitory activity was

Table 4. Sequences of ACE Inhibitory Peptides Isolated on Second HPLC

peak	sequence
P1	Leu-Phe
P3	Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-X
P4	Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe
P6	Gly-Val-Asn-Gly-Glu-Glu-Gly-Val-Pro-Gly

[a] Peak numbers correspond to P1–P6 in **Figure 3B**.

Table 5. ACE Inhibitory Activities of Synthetic Peptides

sequence	IC_{50} (μM)
Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe (P4)	42.4 ± 24.0
Gly-Phe-Pro-Gly-Thr-Pro-Gly-Leu-Pro-Gly-Phe	436.0 ± 160.8
Val-Tyr[a]	26
Val-Pro-Pro[b]	41

[a] Dipeptide from bonito muscle hydrolysate. [b] Tripeptide from milk protein.

measured (**Table 5**). This peptide exhibited an IC_{50} of 42 μM. A peptide, Gly-Phe-Pro-Gly-Thr-Pro-Gly-Leu-Pro-Gly-Phe, was also synthesized by replacing Hyp in P4 with Pro, exhibiting an IC_{50} of 436 μM. This result suggested that Hyp in these peptides played an important role in the ACE inhibitory activity. The inhibitory activity of P4 was compared with that of the known hypotensive peptides. Peptide P4 showed almost the same activity as Val-Tyr and Val-Pro-Pro, which were derived from sardine muscle and milk protein, respectively.

DISCUSSION

There have been many studies on the ACE inhibitory activities of peptides derived from proteins in food. Some of these peptides have been shown to possess hypotensive activity in SHRs. However, there is little information on the ACE inhibitory and hypotensive activities of peptides derived from animal muscle proteins. Therefore, in this study, we examined the hypotensive activity of a chicken extract, because chicken muscle is a popular food throughout the world. We clarified that an extract prepared from chicken breast muscle showed hypotensive activity in SHRs. Furthermore, peptides possessing ACE inhibitory activity were isolated from this extract and their structures were determined.

Thus far, a zein hydrolysate treated with thermolysin has been shown to possess hypotensive activity on intraperitoneal or oral administration to SHRs. The blood pressure was decreased at 1 h after intraperitoneal administration of zein hydrolysate, and this activity continued until 7 h after administration. On the other hand, oral administration of zein hydrolysate exhibited hypotensive activity in SHRs only 6 h later. A hexapeptide derived from casein was reported to have antihypertensive activity and to cause a 10 mmHg reduction in blood pressure at 100 min after an intravenous administration. Although a myofibrillar protein hydrolysate also exhibited hypotensive activity in SHRs on intraperitoneal administration, its activity appeared at 2 h after administration and had disappeared by 4 h (*25*). In our study, the hypotensive activity of a chicken extract in SHRs appeared at 1 h after oral administration and continued for at least 4 h. This is the first observation that hypotensive activity appeared upon oral administration of an animal food material. Furthermore, the hypotensive activity of the chicken extract upon oral administration was also shown to be higher than those of the casein hexapeptide and myofibrillar protein hydrolysate.

Many peptides possessing ACE inhibitory activity have been isolated from various hydrolysates of food proteins. Phe-Phe-Val-Ala-Pro (*11*) and Ile-Pro-Pro (*14*) derived from casein exhibited ACE inhibitory activity (IC_{50} = 2.0 and 5.0 μM, respectively). Val-His-Leu-Pro-Pro (*15*) and Leu-Gln-Pro (*16*), having IC_{50} values of 18 and 9.6 μM, respectively, were also found in a zein hydrolysate. Ile-Trp-His-His-Thr (IC_{50} = 5.1 μM), Ile-Val-Gly-Arg-Pro-Arg-His-Gln-Glu (IC_{50} = 6.2 μM), Ala-Leu-Pro-His-Ala (IC_{50} = 10 μM), Phe-Gln-Pro (IC_{50} = 12 μM), Ile-Tyr (IC_{50} = 3.7 μM), Leu-Lys-Pro-Asn-Met (IC_{50} = 17 μM), Asp-Tyr-Gly-Leu-Tyr-Pro (IC_{50} = 62 μM), and Ile-Lys-Pro-Leu-Asn-Tyr (IC_{50} = 43 μM) were isolated from a thermolysin digest of dried bonito (*17*). An ACE inhibitory peptide was found in a proteolytic hydrolysate of tuna muscle proteins. Met-Asn-Pro (IC_{50} = 66.6 μM), Asn-Pro-Pro (IC_{50} = 290.5 μM), and Thr-Asn-Pro (IC_{50} = 207.4 μM) were discovered in a porcine myosin hydrolysate (*26*). An ACE inhibitory peptide, Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe, isolated in our study exhibited an IC_{50} of 42 μM. This activity was not so high compared with those of peptides derived from casein and zein hydrolysates. However, it exhibited almost the same activity as some peptides derived from dried bonito and a higher activity than peptides derived from porcine myosin. Judging from these results, the chicken extract seems to be useful as a food material possessing antihypertensive activity.

The relationship between the structure and ACE inhibitory activity of peptides remains ambiguous. Cheung et al. (*22*) reported that N-terminal glycyl dipeptides vary 300-fold in inhibitory potency, those with Trp, Tyr, Pro, or Phe being the most inhibitory. Although many of the inhibitory peptides described above certainly possessed Pro, Tyr, or Phe at their C terminus, peptides without them at the C terminus also exhibited high activity. The rule proposed by Cheung et al. may apply to evaluation of the activities of short peptides such as di- and tripeptides. However, ACE exhibits relatively broad substrate specificity like peptidyldipeptidase, which hydrolyzes bradykinin, a nanopeptide, and angiotensin I, a decapeptide. Furthermore, in our work, the substitution of Pro for Hyp in Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe decreased the inhibitory activity 10-fold, indicating that the hydroxyl group of Hyp in peptides plays an important role in the binding of peptides to the catalytic site of ACE. The amino acid sequences at the N termini of oligopeptides also seem to significantly influence their ACE inhibitory activities. The relationship between the N-terminal structure and ACE inhibitory activity is the next problem to be resolved.

Homology analysis of three isolated peptides, Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe, Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-X, and Gly-Val-Asn-Gly-Glu-Glu-Gly-Val-Pro-Gly, has shown that these peptides are derived from collagen in chicken muscle. Oshima et al. (*8*) reported that ACE inhibitory peptides were found in digests of gelatin with bacterial collagenase, possessing the structures of Gly-Pro-Hyp-Gly-Thr-Asp-Gly-Ala-Hyp (IC_{50} = 10.5 μM), Gly-Pro-Ala-Gly-Ala-Hyp (IC_{50} = 8.3 μM), Gly-Pro-Pro-Gly-Ala-Hyp (IC_{50} = 8.6 μM), Gly-Pro-Ile-Gly-Ser-Val-Gly-Ala-Hyp (IC_{50} = 31.8 μM), and Gly-Pro-Ala-Gly-Ala-Pro-Gly-Ala-Ala (IC_{50} = 37.0 μM). The structures of the peptides in our study were different from those of peptides in gelatin digests. These differences in structure seemed to be caused by the differences in the origin of collagen, because it is well-known that there are many types of collagens, which have heterogeneous structures.

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