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Angiotensin I-Converting Enzyme-Inhibitory Peptides Obtained from Chicken Collagen Hydrolysate

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In this study, collagen extracted from chicken legs (which are the yellow keratin parts containing a nail) was hydrolyzed with various enzymes, and the angiotensin I-converting enzyme (ACE)-inhibitory activity of each hydrolysate was determined. The hydrolysate by treatment with an *Aspergillus* species-derived enzyme had the highest activity ($IC_{50} = 260$ μg/mL). The fraction of this hydrolysate obtained by ultrafiltration with a molecular-weight cutoff of 3000 Da (low fraction) had a stronger activity ($IC_{50} = 130$ μg/mL) than the fractionated one. This fraction was further fractionated by HPLC, and the peptides in the fraction with high ACE-inhibitory activity were identified. The amino acid sequences of the four peptides were identified using a protein sequencer. These peptides were synthesized to confirm their ACE-inhibitory activities; this showed that peptides with a Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro sequence had the highest activity ($IC_{50} = 29$ μM). When the low fraction was administered to spontaneous hypertensive rats, a decrease in their blood pressure was observed after 2 h of administration, and a significant decrease in blood pressure (−50 mmHg) was observed after 6 h. Moreover, long-term administration studies indicated that the low fraction showed a significant suppression of increased blood pressure.

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

INTRODUCTION

High blood pressure is thought to be the main risk factor of cardiovascular disease and stroke. It is reported that the estimated total number of adults with hypertension is ∼1 billion in the world (*1*). Therefore, the study of the prevention of hypertension by functional foods is a very important project.

Recently, some components derived from foods, including dried bonito (*2*), sardines (*3*), skipjack tuna (*4*), and wakame seaweed (*5*) have been shown to have an antihypertensive effect; some of these materials are marketed as food products for persons with a raised blood pressure. However, these functional foods for a person with high blood pressure are not fully studied and are not necessarily applied.

We have previously reported that chicken breast muscle hydrolysate possesses an antihypertensive effect, and collagen-derived angiotensin I-converting enzyme (ACE)-inhibitory peptides were isolated as the effective entity (*6, 7*). However, it is not efficient to make functional peptides that extract the collagen from chicken breast muscle. Therefore, we used chicken legs, which are the yellow keratin parts, as a source of collagen because they are discarded in many cases.

Although chicken legs contain a large amount of type I collagen, it is difficult to turn this purified collagen into food products because of its yellow color and unique smell. We extracted collagen from chicken legs with a hot aqueous acid solution and decolorized and deodorized the extracted collagen by treating it with activated carbon. In addition, the collagen was hydrolyzed with an enzyme derived from an *Aspergillus* species, and a fraction with a molecular weight of 6000 Da or less was separated using an ultrafiltration membrane for easier handling and higher digestivity. These purified chicken collagen peptides were found to transfer into blood 2 h after ingestion (*8*), and peptides with a lower molecular weight were shown to have a lower allergenicity. However, other functions of this chicken collagen peptide have not been studied thoroughly. The antihypertensive effect of a product consisting of chicken collagen peptides was examined as part of a study of its functionality.

In this study, we prepared a chicken collagen hydrolysate possessing a strong ACE-inhibitory activity and examined the tolerance of its activity after digestive enzyme treatment. Furthermore, the hypotensive activity by administering the hydrolysate to spontaneously hypertensive rats (SHRs) was

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confirmed simultaneously. Control of blood pressure by means of diet is considered to be very important; therefore, the objective of this study was to contribute to the widening of the range of foods that are appropriate for hypertensive patients through elucidation of the antihypertensive effect of chicken collagen peptides.

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). Protease FP and Protease N were purchased from Shin Nihon Chemical Co. (Aichi, Japan), and A amano G was from Amano Enzyme Co. (Aichi, Japan). Other chemical reagents were of reagent grade or better.

Preparation of Chicken Collagen Hydrolysate. Chicken collagen hydrolysate was made from chicken legs according to the following method. One kilogram of chicken legs was boiled in 1.5 L of aqueous solution to extract the collagen adequately. After boiling, the extract was filtered and centrifuged to remove the precipitate. Furthermore, the supernatant was hydrolyzed with 0.1% *Aspergillus oryzae* protease, which has a broad substrate specify (Mitsubishi-kagaku Food Co., Tokyo, Japan) to lower the viscosity of the product. Furthermore, it was applied to an ultrafiltration membrane with a cutoff molecular weight of >6000 Da (Asahikasei Chemical Co., Tokyo, Japan). This hydrolysate was eluted on an activated carbon column for decolorization and deodorization.

Digestion of Chicken Collagen Hydrolysate with Proteases. The chicken collagen hydrolysate was treated with 0.1% proteases (protease FP, protease A amano G, and Protease N) at 50 °C and pH 7.0 for 4 or 24 h. These proteases are a mixture of endo- and exo-type peptidases with a broad substrate specificity. After this reaction was stopped by boiling for 10 min, the supernatant was further hydrolyzed by successive treatment with 1% pepsin and trypsin/chymotrypsin at 37 °C and pH 7.0 for 1 h, respectively. Each digestion also was stopped by boiling for 10 min to inactivate the proteases. After treatment, all hydrolysates were centrifuged to remove the precipitates. Porcine collagen hydrolysates also were prepared in same manner.

Separation and Purification of ACE-Inhibitory Peptides from Chicken Collagen Hydrolysate. Peptides in a chicken collagen hydrolysate were first divided into two groups, with molecular masses of less and more than 3000 Da, respectively, using an ultrafiltration membrane (Millipore Co., Bedford, MA). Peptides in the former group possessing a high ACE-inhibitory activity were purified by HPLC on octadecyl silane (ODS) columns (22 mm × 250 mm and 4.6 mm × 250 mm; TOSOH, Tokyo, Japan) using a linear gradient of CH_3CN (8−40% in 40 min or 8−40 % in 64 min, respectively) containing 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min. The elution peaks were monitored at 220 nm.

Measurement of Blood Pressure 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 and 55% humidity), and then either saline or a hydrolysate (3 g/kg wt) dissolved in saline was administered. Their tail systolic blood pressure and heartbeat were determined by a tail-cuff method using a plethysmographic tail apparatus (Softron 98A; Softron Co., Tokyo, Japan). Student's *t*-test was used to analyze as to whether there were significant differences among the data. All the animal procedures in this study complied with the standards set out in the Guideline for the Care and Use of Laboratory Animals at Nippon Meat Packers, Inc.

Assaying of Inhibitory Activity toward ACE. The inhibitory activity of peptides toward ACE was assayed according to the method reported by Cheung (*10*). 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 rabbit lung ACE, and 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

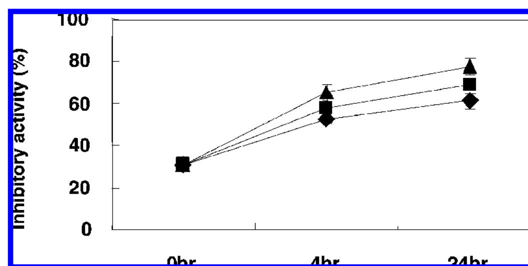

Figure 1. Comparison of ACE-inhibitory activity of chicken collagen hydrolysates treated by each enzyme: ▲, protease FP; ■, protease A amano G; and ♦, protease N. Data represent mean ± SEM (n = 3).

Table 1. ACE-Inhibitory Activity of Each Chicken Collagen Hydrolysate[a]

sample	nontreated fraction	low fraction (<3000 Da)	high fraction (>3000 Da)
IC_{50} = (mg/mL)[b]	0.26 ± 0.02	0.13 ± 0.01	0.28 ± 0.02

[a] Data represent mean ± SEM (n = 3). [b] Concentration of peptide needed to inhibit 50% of ACE activity.

the released hippuric acid at 228 nm after successive extraction with ethyl acetate, evaporation of 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 previously was expressed as IC_{50}, which was calculated using the net weight of the protein hydrolysate.

Analysis of 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. Four kinds of peptides that were isolated from hydrolysate as potent ACE-inhibitory 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. (*11*). After being synthesized, a peptide was purified by HPLC on an ODS column (PEGASIL-300, 20 mm × 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 monitored at 220 nm). Furthermore, the molecular mass of the isolated peptide was determined by mass spectrometry with an ESI mass spectrometer LC-Q (Thermo Finnigan, San Jose, CA).

Statistical Evaluations. All data are presented as the mean ± SEM for the number of determinations shown in the figures or tables. Data on blood pressure were evaluated by one-way ANOVA, followed by Student's *t*-test to compare the mean of each dose group to that of the control group. The probability level used to determine the statistical significance was $p < 0.05$.

RESULTS

ACE-Inhibitory Activities of Chicken Collagen Hydrolysates. Figure 1 shows the ACE-inhibitory activities of chicken collagen hydrolyzed with various enzymes. Chicken collagen hydrolysates inhibited ∼30% of the activity, whereas further enzymatic treatment doubled their activities. Among the various enzymatic treatments, treatment with the FP enzyme provided the hydrolysates with the highest activity.

Peptides treated with FP enzyme were then fractionated by using an ultrafiltration membrane with a molecular-weight cutoff of 3000 Da. The magnitudes of the ACE-inhibitory activities of the peptides were then compared to each other. This revealed that the peptide with lower molecular weights had higher activities (**Table 1**).

To compare the ACE-inhibitory activities of collagen hydrolysate from another species, porcine collagen hydrolysates

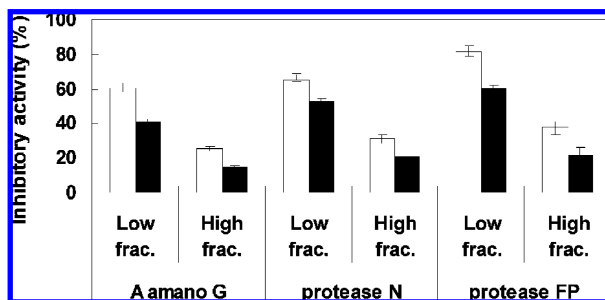

Figure 2. Comparison of ACE-inhibitory activity toward chicken and porcine collagen hydrolysates (1 mg/mL). Each hydrolysate was fractionated by an ultrafiltration cutoff of 3000 Da. Low fraction and high fraction represent molecular weights under and over 3000 Da, respectively. □: Chicken collagen hydrolysate and ■: porcine collagen hydrolysate. Data represent mean ± SEM (n = 3).

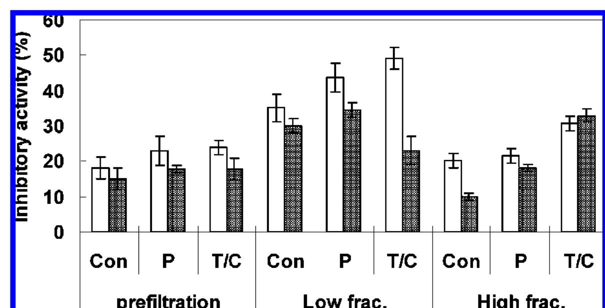

Figure 3. Tolerance of ACE-inhibitory acitivity toward gastric enzyme treatment (0.5 mg/mL). P and T/C represent hydrolysates prepared by pepsin and trypsin/chymotrypsin. Low fraction and high fraction represent molecular weights under and over 3000 Da, respectively. □: Chicken collagen hydrolysate and ■: porcine collagen hydrolysate. Data represent mean ± SEM (n = 3).

were prepared in the same manner (**Figure 2**). As in the case of chicken, hydrolysates treated with the FP enzyme had the highest activity, which had a similar magnitude as that of the hydrolyzed collagen from chicken.

Effect of Treatment with Gastrointestinal Enzymes on ACE-Inhibitory Activity. Next, a model digestive system was used to determine as to whether the ACE-inhibitory activity was retained after the ingestion of chicken or porcine collagen hydrolysate (FP treatment) into the body (**Figure 3**). The activity of chicken-derived peptides increased following treatment with digestive enzymes, whereas that of porcine-derived peptides decreased when their molecular weight was lowered by treatment with digestive enzymes.

Antihypertensive Effect in SHRs. The blood pressures of SHRs were measured after oral administration of FP treatment. A reduction in blood pressure was observed 4 h after administration in SHRs administered a low molecular weight fraction of chicken collagen hydrolysate (low fraction), as compared to SHRs administered saline, and the lowest blood pressure was observed 8 h after administration (**Figure 4A**). Long-term administration studies showed that there was a reduction in blood pressure in SHRs administered the low fraction for 1 week, and a significant reduction in blood pressure was observed after a 2 week administration ($p < 0.05$) (**Figure 4B**). Although serum minerals, including sodium, potassium, and calcium, are known to affect blood pressure, no differences were found in the levels of these minerals between a control group and a group given the low fraction, even after a 4 week administration (**Table 2**).

Purification of ACE-Inhibitory Peptides. The ACE-inhibitory peptides were isolated by reversed-phase high-performance

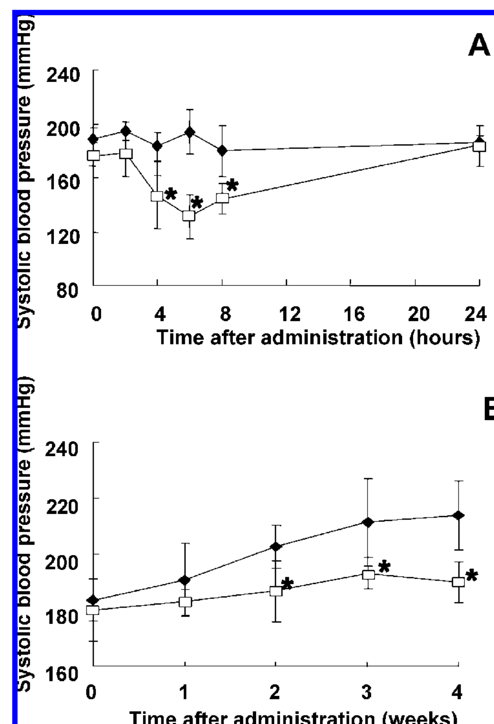

Figure 4. Changes in blood pressure upon oral administration of chicken collagen hydrolysate. (**A**) Single administration of chicken collagen hydrolysate and (**B**) long-term administration of chicken collagen hydrolysate. □: Chicken collagen hydrolysate and ♦: saline (control). Av blood pressure values in five SHRs are shown. *: Significantly different from the control (*, $p < 0.05$) with Student's t-test.

Table 2. Analysis of Blood Component

blood component	unit	control group	peptide group
Na	mequiv/L	144.5 ± 20.3	143.8 ± 18.4
K	mequiv/L	3.78 ± 0.4	3.75 ± 0.5
Cl	mequiv/L	102.8 ± 12.1	102.3 ± 10.8
Ca	mg/dL	9.2 ± 1.5	9.23 ± 1.7
hydroperoxide	nmol/mL	1.03 ± 0.3	0.97 ± 0.2
LDL-cho	mg/mL	7.25 ± 1.5	8.75 ± 1.4
adrenaline	ng/mL	4.5 ± 0.8	3.18 ± 0.6
noradrenaline	ng/mL	0.68 ± 0.2	0.94 ± 0.3
dopamine	ng/mL	0.06 ± 0.02	0.05 ± 0.02

Data represent mean ± SEM (n = 5).

liquid chromatography (HPLC) of the low fraction with an elusion rate (1 %/min) by using acetonitrile (**Figure 5A**). Six fractions (fractions 1−6) with different elution times were obtained, and their inhibitory activities were compared (**Table 3A**). Fraction 4, which showed a strong activity, was collected and subjected to a second HPLC fractionation with a slower elution rate (0.4 %/min) (**Figure 5B**). The peaks obtained were further divided on the basis of their elution times (fractions 4-1−4-4), and the ACE-inhibitory activities of these fractions were compared (**Table 3B**). Fraction 4-3, which showed the highest activity, was recollected and injected into the HPLC instrument at a slower elution rate (0.16 %/min) by using acetonitrile (**Figure 5C**). The amino acid sequences of the resulting fractions were determined by using a protein sequencer.

Structure Analysis of ACE-Inhibitory Peptides. The amino acid sequences of the resulting peptides (**Figure 5C**) and the data of their molecular masses are shown in **Table 4**. Their structures originated from the chicken collagen α-chain and the

Figure 5. Isolation of ACE-inhibitory peptides from chicken collagen hydrolysate. (**A**) First fractionation of chicken collagen hydrolysate; (**B**) second fractionation of fraction 4 in panel **A**; and (**C**) third fractionation of fraction 4-3 in panel **B**.

relatively long peptides of 8mers to 16mers. Moreover, the sequences entered with a concentration of 1000−2000 ppm into the hydrolysate. Peptide 8 showed the strongest ACE-inhibitory activity (IC_{50} = 29.4 μM).

DISCUSSION

In this study, we demonstrated that low molecular-weight fractions obtained from chicken collagen hydrolysate by ultrafiltration with a molecular-weight cutoff of 3000 Da showed an antihypertensive effect on SHRs. Among these fractions, we identified four peptides with an ACE-inhibitory activity, such as Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro, Gly-Ala-Hyp-Gly-Pro-Ala-Gly-Pro-Gly-Gly-Ile-Hyp-Gly-Glu-Arg-Gly, Gly-Leu-Hyp-Gly-Ser-Arg-Gly-Glu-Arg-Gly-Leu-Hyp-Gly, and Gly-Ile-Hyp-Gly-Glu-Arg-Gly-Pro-Val-Gly-Pro-Ser-Gly, and their IC_{50} values were 29.4, 45.6, 60.8, and 43.4 μM, respectively.

Table 3. ACE-Inhibitory Activity of Each Fraction

(**A**) First Fractionation

sample	fraction 1	fraction 2	fraction 3	fraction 4	fraction 5	fraction 6
IC_{50} (mg/mL)[a]	0.17 ± 0.005	0.262 ± 0.004	0.300 ± 0.001	0.139 ± 0.003	0.173 ± 0.002	ND

(**B**) Second Fractionation by HPLC

sample	fraction 4-1	fraction 4-2	fraction 4-3	fraction 4-4
IC_{50} (mg/mL)[a]	0.110 ± 0.002	0.105 ± 0.003	0.075 ± 0.001	ND

[a] Concentration of peptide needed to inhibit 50% of ACE activity. Data represent mean ± SEM ($n = 3$).

Table 4. Sequences and Mass Spectrometry of ACE-Inhibitory Peptide[a]

peptide no.	mass	sequence	homology	concentration (ppm)	IC_{50} (μg/mL)
3	ND	Ala and His			
4	ND	Asp, Gly, and Phe			
5	ND	Trp			
6	ND	amino acids (mixture)			
7	ND	ND			
8	697.4	Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro	collagen α1	1500	29.4 ± 3.1
9	1378.9	Gly-Ala-Hyp-Gly-Pro-Ala-Gly-Pro-Gly-Gly-Ile-Hyp-Gly-Glu-Arg-Gly	collagen α2	1140	45.6 ± 2.2
10	ND	ND			
11	1284.8	Gly-Leu-Hyp-Gly-Ser-Arg-Gly-Glu-Arg-Gly-Leu-Hyp-Gly	collagen α2	950	60.8 ± 4.3
12	1494.8	Gly-Ile-Hyp-Gly-Glu-Arg-Gly-Pro-Val-Gly-Pro-Ser-Gly	collagen α2	2050	43.4 ± 1.5

[a] Peptide numbers correspond to **Figure 4C**.

It was clarified that various food hydrolysates possess an ACE-inhibitory activity. Wheat (*12*), soybean (*13*), salmon (*14*), etc. are reported to be potent ACE-inhibitory sources. Among them, sour milk (*15*), bonito (*2*), and sardine (*16*) are commercialized as food for specified health uses and have received the approval of the Ministry of Health, Labour and Welfare of Japan.

As for the structure−activity correlations between ACE and ACE-inhibitory peptides, it is known that the peptides containing hydrophobic (aromatic or branched side chain) amino acid residues at the three C-terminal positions possess a strong ACE-inhibitory activity. In this study, chicken collagen hydrolysate prepared with protease FP showed a high inhibitory activity (**Figure 1**). Furthermore, this activity was raised by gastric enzymes. We speculated that the peptides in chicken collagen hydrolysate were broken down into small fragments possessing hydrophobic amino acids at the C-terminus by gastric enzymes such as chymotrypsin. In addition, no difference in the ACE-inhibitory activity was observed between the peptides obtained from chicken collagen by enzymatic treatment and those obtained from porcine collagen, whereas hydrolysates obtained from chicken collagen treated with an internal digestive enzyme showed a higher activity. This may be because the amino acid composition of porcine collagen is more similar to that of human collagen (*17*) and is more susceptible to digestion by internal digestive enzymes, so that the peptide becomes free amino acids that do not show an ACE-inhibitory activity. Alternatively, chicken collagen may have an amino acid sequence that intrinsically shows a greater ACE-inhibitory activity.

Single oral administration of low molecular-weight chicken collagen hydrolysates significantly lowered the blood pressure in SHRs, and long-term oral administration even suppressed the hypertension in these animals (**Figure 4**). It is thought that ACE-inhibitory peptides or potent inhibitory peptides in chicken collagen hydrolysate show hypotensive activity through gastrointestinal digestion and absorption.

In this experiment, four peptides were isolated as ACE-inhibitory peptides. There have been many studies concerning ACE-inhibitory peptides derived from food proteins, but only a few of these relate to meat and meat products. Arihara et al. reported ACE-inhibitory peptides, Ile-Thr-Thr-Asn-Pro and Met-Asn-Pro-Pro-Lys, from the thermolysin digest of porcine myosin, and their IC_{50} values were 549.0 and 945.5 μM, respectively (*18*). A 9mer peptide (Arg-Met-Leu-Gly-Gln-Tyr-Pro-Tyr-Lys; $IC_{50} = 34$ μM) was isolated from porcine toroponin C with pepsin treatment (*19*). Relatively strong ACE-inhibitory peptides were found in chicken muscle hydrolysate digested with thermolysin, such as Phe-Gln-Lys-Pro-Lys-Arg and Ile-Lys-Trp ($IC_{50} = 14$ and 0.21 μM, respectively) (*20*). Although nine peptides obtained from gelatin also were reported to be ACE-inhibitory peptides (*21*), the amino acid sequence of peptides reported here is novel and different from those previously reported. Furthermore, the ACE-inhibitory activity of chicken collagen peptides (IC_{50} values ranging from 29.4 to 60.8 μM) was equivalent or stronger than those activities described previously, and the activity also was stronger than chicken breast muscle collagen peptide, which we reported previously ($IC_{50} = 42$ μM) (*6*).

Chicken collagen hydrolysate prepared in this study was composed of foods that can be easily incorporated into the daily diet. It is important to prevent and improve hypertension, which is a lifestyle-related disease, in the course of daily life. By incorporating these foods into meals, normalization of blood pressure will be achieved without compromising the quality of life of those who need such foods.

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