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ARTICLE

Novel Angiotensin-Converting Enzyme (ACE) Inhibitory Peptides Derived from Boneless Chicken Leg Meat

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Four peptides that inhibit angiotensin-converting enzyme (ACE) were separated from the hydorlysate of boneless chicken leg meat digested with artificial gastric juice (pepsin). Two peptides were identified as the peptides encrypted in myosin heavy chain. The peptide P1 (MNVKHWPWMK) corresponds to the amino acid sequence from amino acids 825 to 834 of myosin heavy chain, and the peptide P4 (VTVNPYKWLP) corresponds to the amino acid sequence from amino acids 125 to 135 of myosin heavy chain. They are novel ACE inhibitory peptides derived from chicken, and IC_{50} values of P1 and P4 were determined as 228 and 5.5 μM, respectively. Although these values were much larger than 0.022 μM for captopril, a typical synthetic ACE inhibitor, they are comparable to IC_{50} values reported for various ACE inhibitory peptides derived from foods. Because the peptide P4 has a relatively low IC_{50} value, it is a good starting substance for designing food supplements for hypertensive patients.

KEYWORDS: Angiotensin-converting enzyme inhibitors; peptide; myosin; chicken

INTRODUCTION

Recent studies have revealed various functional roles of the peptides derived from foods on the physiological regulation of humans (*1*, *2*). Such peptides are attributed to active peptide encrypted in protein molecules and are generated by protease action in digestion processes. While milk and egg are particular sources of such peptides, the physiologically active peptides have also been found in various kinds of meats and plants. These peptides show a number of different activities in cardiovascular, endocrine, immune, and nerve systems. Among those physiological effects of the peptides, antihypertensive effects have attracted the attention of researchers for a decade (*3*, *4*), because hypertensive patients are susceptible to heart attack and ischemic cardiac disease, and these cardiovascular diseases are the second most common cause of death in many developed countries.

The renin−angiotensin system is one of the important mechanisms regulating blood pressure (*5*, *6*). Briefly, an enzyme renin, secreted from the kidney, generates angiotensin I by cleaving angiotensinogen. Angiotensin I (DRVYIHPHL) is then converted to angiotensin II, which has a strong vasoconstrictive effect, with anigotensin-converting enzyme (ACE) by cleaving HL in the C-terminal end. Further, ACE also cleaves bradykinin, a peptide that has a pronounced vasodilating effect. Therefore, inhibition of ACE activity is effective to reduce blood pressure, and various ACE inhibitory peptides have been isolated from collagenase hydrolysate of gelatin (*7*), trypsin hydrolysate of casein (*8*), and thermal hydrolysate of tuna (*9*), corn (*10*), soybeans (*11*), sardine muscle (*12*), and dried bonito (*13*). The strength of those peptides is evaluated by the IC_{50} value, the peptide concentration that inhibits 50% of ACE activity. While the IC_{50} value of captopril, a typical synthetic ACE inhibitor used as a drug for hypertensive patients is reported to be 0.022 μM (*14*), the peptides derived from food proteins show IC_{50} values from 0.1 μM to a few hundred micromolar, depending upon their amino acid sequences. Nonetheless, the control of the blood pressure with the ACE inhibitory peptides derived from food proteins has been extensively studied (*15*, *16*), because the synthetic ACE inhibitors are known to have strong side effects, such as cough, skin rashes, and angioedema (*17*). Antihypertension effects of these peptides have been demonstrated using spontaneously hypertensive rats (SHRs) with oral administration (*18*−*20*). Some of such antihypertensive peptides are already commercialized. For example, the lactotripeptides, VPP and IPP, are marketed as a dietary supplement for hypertensive patients.

We have characterized four ACE-inhibitory peptides generated from bonito proteins by pepsin digestion (*21*−*23*). Further, we have successfully produced an ACE inhibitory peptide found in the bonito proteins, PTHIKWGD, using *Escherichia coli* as a host strain (*24*). In this work, we have studied the generation of ACE inhibitory peptides from chicken leg meat by pepsin digestion to elucidate health effects of consuming the chicken meats. We have identified four novel ACE inhibitory peptides from pepsin hydrolysate of boneless chicken leg meat. Two peptides are identified as the peptides encrypted in myosin heavy chain. Further, IC_{50} values of these peptides have been determined using synthetic peptides.

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MATERIALS AND METHODS

Materials. ACE and pepsin were purchased from Sigma-Aldrich, Japan. Hippuryl-L-histdyl-L-leucine (HHL) and Cosmosil $5C_{18}$-MS-II (4.6 × 150 mm) were purchased from Nacalai Tesque, Inc. (Japan). Synthetic peptides were purchased from Hokkaido System Science Co., Ltd. (Japan). All other reagents used in this work were of reagent grade.

Digestion of Boneless Chicken Leg Meat with Artificial Gastric Juice. Boneless chicken leg meat (17.9 g) purchased at a local market was boiled for 6 min and then grayed with a motor and pestle. The grayed meat was added to 200 mL of artificial gastric juice (5.0 mg/mL pepsin and 30 mM NaCl), and pH of the solution was adjusted to pH 2.0 with HCl. After the solution was divided into five aliquots, the aliquots were incubated at 37 °C. At time 0, 15, 30, 45, and 60 min, one aliquot was taken out to adjust the pH to pH 7.0 with 1 M NaOH for inactivating pepsin. The hydrolysate was then filtrated with Centriprep YM-30 (Amicon, M_w cutoff of 30 000) for further analysis.

ACE Activity Measurement. The activity of ACE was measured as described in the previous paper (*23*). Briefly, 25 μL of phosphate buffer (50 mM KH_2PO_4 at pH 8.3), 10 μL of hydrolysate, and 10 μL of ACE solution (0.2 units/mL, 50 mM KH_2PO_4 at pH 8.3) were mixed and preincubated for 10 min at 37 °C. The preincubation was carried out to digest the non-specific proteins and peptides coexisting in the hydrolysate by ACE. After 25 μL of HHL solution (8.3 mM HHL, 133 mM KH_2PO_4, and 500 mM NaCl) was added, the reaction mixture was further incubated for 30 min at 37 °C. The ACE reaction was terminated by adding 70 μL of 1 M HCl. After the reaction mixture was filtrated with Millex-LG (Miliipore Corp.), 20 μL of the reaction mixture was injected to a high-performance liquid chromatography (HPLC) system (Shimadzu LC-10, Japan) equipped with a hydrophobic column (Cosmosil 5C18-MS-II, 4.6 × 150 mm). An isocratic mobile phase was 80% Milli-Q water containing 0.1% (v/v) trifluoroacetic acid and 20% acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The flow rate was 1.0 mL/min. Hippuric acid (HA) generated by the ACE reaction and the unreacted HHL were detected at 228 nm with a spectrophotometer (Shimadzu SPD-20A). Retention times of HA and HHL were 4.5 and 27 min, respectively.

The inhibition percentage of the ACE was defined by the following equation:

$$\text{ACE inhibition (\%)} = \{[(\text{HA peak height})_{\text{without inhibitor}} - (\text{HA peak height})_{\text{with inhibitor}}]/(\text{HA peak height})_{\text{without inhibitor}}\} \times 100$$

Determination of IC_{50}. The intensity of the inhibitory effect of the peptides was evaluated by the IC_{50} value. The ACE reaction rates were measured with the different peptide concentrations, and $[(V_m - V_{in})/V_{in}]$ was plotted against the peptide concentration on a logarithmic scale, where V_m is the reaction rate without the inhibitor and V_{in} is the reaction rate with the inhibitor. Because a competitive inhibition model can be applied to the ACE inhibition with the inhibitory peptides, this plot should give a straight line with the slope of 1.0.

Separation of Peptides with HPLC. The peptides generated in the hydrolysate were separated with a HPLC system equipped with a hydrophobic column (Cosmosil 5C18-MS-II, 4.6 × 150 mm). Gradient elution with solution A [Milli-Q water containing 0.1% (v/v) trifluoroacetic acid] and solution B [acetonitrile containing 0.1% (v/v) trifluoroacetic acid] was applied to separate the generated peptides. Dependent upon the experimental purpose, 50 min gradient program (B 30% at 30 min, B 50% at 34 min, B 0% at 47 min) or 70 min gradient program (B 30% at 30 min, B 50% at 60 min, B 0% at 65 min) was used. The effluent was monitored at 215 nm with a spectrophotometer (Shimadzu diode array detector, SPD-M10AVP). Some portion of the effluent from the spectrophotometer was fractionated for further analysis. While 20 μL of the sample was injected for analytical purpose, 200 μL of the sample was applied to HPLC for the fractionation. The fractionated samples were freeze-dried, and were dissolved with Milli-Q water.

RESULTS AND DISCUSSION

ACE inhibition (%) of the boneless chicken leg meat digested with the artificial gastric juice is shown in **Figure 1**. The ACE inhibition (%) increased with the increase of the digestion time, which clearly shows that the ACE inhibitory peptides were generated from the boneless chicken leg meat by digestion with the artificial gastric juice. The sample digested for 60 min was used for further analysis, because it showed the highest value. **Figure 2** shows a chromatogram of this sample separated with HPLC using the 50 min gradient program. The effluent from the spectrophotometer was fractionated every 10 min, and the ACE inhibition (%) of the fractions was determined as summarized in **Table 1**. Because the fraction collected from 30 to 40 min showed the strongest inhibition, this portion was further separated with HPLC using the 70 min gradient program. A chromatogram from 30 to 40 min for the same sample is shown in **Figure 3**, and the ACE inhibition (%) of the fractions collected every 2 min is shown in **Table 2**. The fraction collected from 34 to 36 min showed the highest value (97.8%).

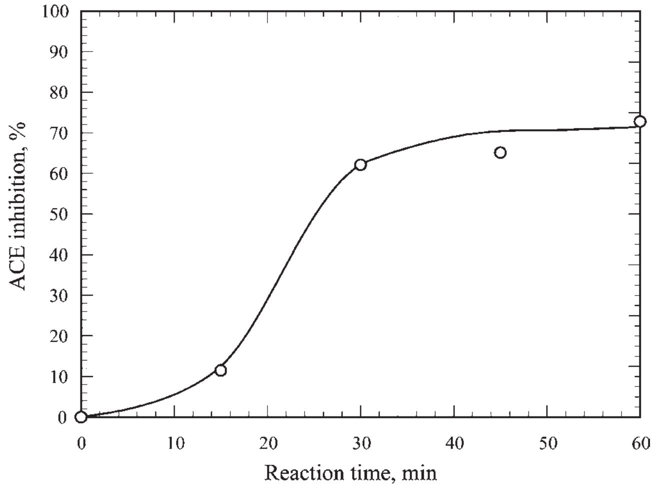

Figure 1. Change of ACE inhibition (%) of hydrolysate with the reaction time.

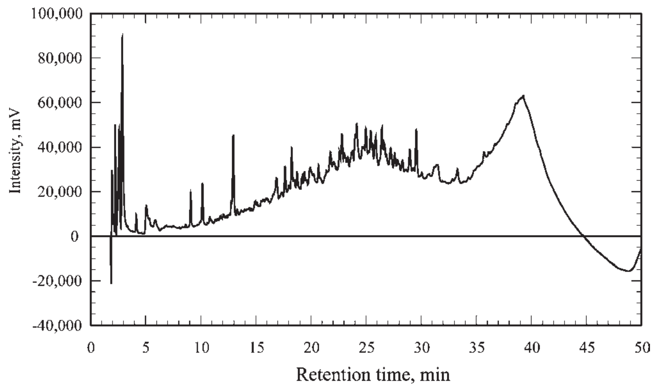

Figure 2. Chromatogram of hydrolysate digested for 60 min with pepsin. Peptides were separated with a 50 min gradient program (see the Materials and Methods for details).

Table 1. ACE Inhibition (%) of the Fractionated Sample (50 min Gradient Program)

time (min)	0−10	10−20	20−30	30−40	40−50
ACE inhibition (%)	23.4	28.1	30.7	49.4	41.1

Because the four independent peaks were observed in this period (peaks 1−4 shown in **Figure 3**), these peaks eluted from the spectrophotometer were collected separately. This fractionation was carried out 9 times, and then the collected fractions were freeze-dried and dissolved in 135 μL of Milli-Q water. Amino acid sequences of the peptides (P1−P4) in the fractions were

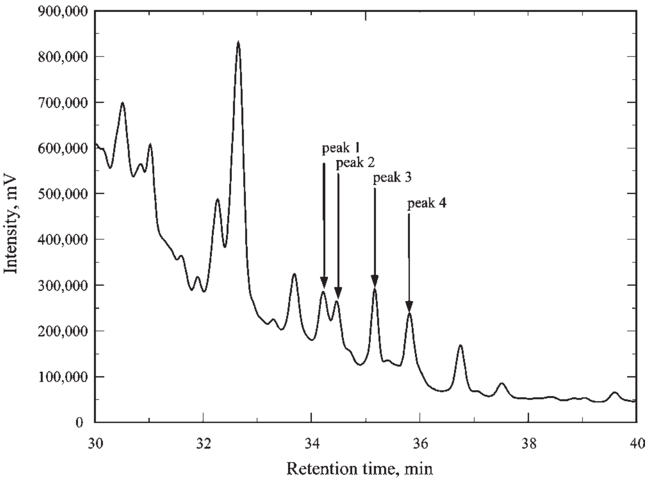

Figure 3. Chromatogram of hydrolysate digested for 60 min with pepsin. Peptides were separated with s 70 min gradient program (see the Materials and Methods for details).

Table 2. ACE Inhibition (%) of the Fractionated Sample (70 min Gradient Program)

time (min)	30−32	32−34	34−36	36−38	38−40
ACE inhibition (%)	81.9	78.5	97.8	31.7	nd[a]

[a] nd = not detected.

Table 3. Amino Acid Sequence of Identified Peptides

peak	amino acid sequence
P1	MNVKHWPWMK
P2	INDNFYDWLP
P3	WDWPY
P4	VTVNPYKWLP

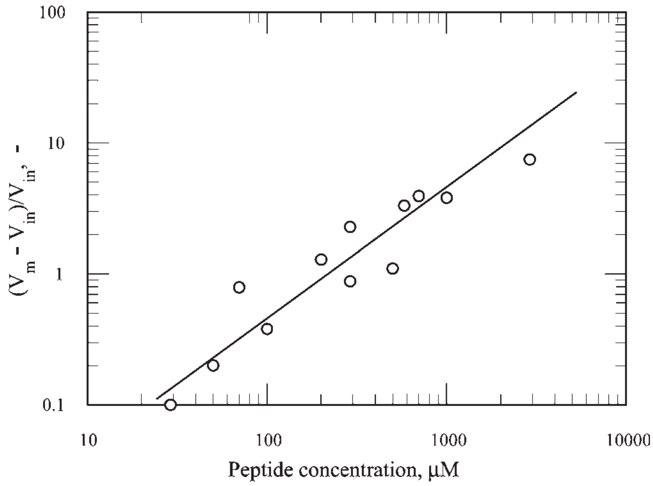

Figure 4. Determination of IC_{50} for peptide P1.

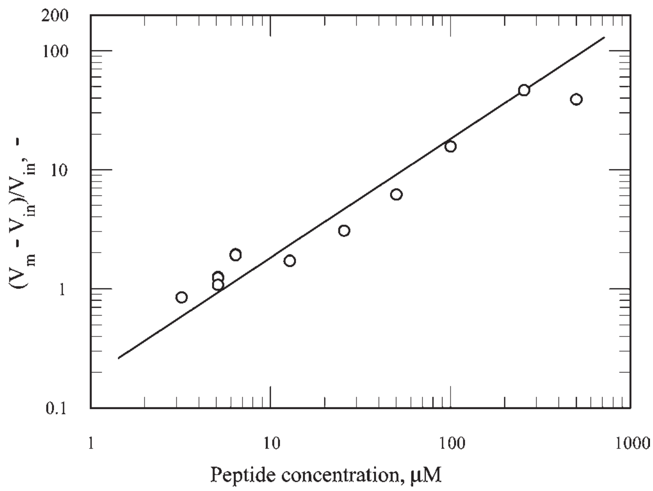

Figure 5. Determination of IC_{50} for peptide P4.

determined with a peptide sequencer (ABI Procise492, Applied Biosystems, Carlsbad, CA), and their amino acid sequences are shown in **Table 3**. Peptide 1 (P1) and petide 4 (P4) were identified as the peptides derived from chicken myosin heavy chain (P1, from amino acids 825 to 834; P4, from amino acids 125 to 135). Unfortunately, the amino acid sequences of P2 and P3 were not found in the chicken proteins reported in database Uniprot (http://www.uniprot.org/).

To determine IC_{50} values of peptides P1 and P4, the ACE inhibition (%) was determined using synthetic peptides. $[(V_m - V_{in})/V_{in}]$ was plotted against the peptide concentration in the double-logarithmic charts (**Figure 4** for P1 and **Figure 5** for P4). The straight lines in **Figures 4** and **5** were drawn with the least-squares approximation. Because the IC_{50} value is the peptide concentration that gives $[(V_m - V_{in})/V_{in}] = 1.0$, IC_{50} values were determined as 228 μM for P1 and 5.5 μM for P4. Although these values are much larger than 0.022 μM for captopril, they are comparable to the IC_{50} values reported for various ACE inhibitory peptides derived from foods. For example, VPP and IPP, commercialized as a dietary supplement from Calpis Co., Ltd. (Japan), have IC_{50} values of 9 and 5 μM, respectively (*25*).

The ACE inhibitory peptides derived from porcine and chicken meats are summarized in **Table 4**. Despite extensive studies by many researchers, however, any common sequence for strong ACE inhibitory peptides has not yet been found. To our knowledge, the peptides P1 and P4 are novel ACE inhibitory peptides encrypted in chicken myosin heavy chain. It should be noted that the IC_{50} value of the peptide P4 is much smaller than most of those reported in **Table 4**. Because the effectiveness of various peptides for reducing blood pressure is clinically proven, the peptide P4 (IC_{50} value of 5.5 μM) is expected to be effective *in vivo*.

While the peptides P1 and P4 are longer than the ACE inhibitory peptides reported in the literature, much shorter peptides are preferable because the peptides longer than three amino acids are considered not to be taken up from the intestines and, further, the binding of the peptides to ACE would be improved for the shorter peptides. The peptides P1, P2, P3, and P4 are good starting materials for designing the potent ACE inhibitory peptides without side effects. Currently, the ACE inhibitory activities studied for the synthetic peptides consist of three amino acids encrypted in the peptides found in this work.

As for the health effects of consuming chicken meat, different approaches are required. The changes in peptide lengths and ACE inhibitory activities of the peptides found in this work by the hydrolysis with the intestinal proteases, such as trypsin and chymotrypsin, should be clarified. Purification of other ACE inhibitory peptides from the hydrolysates digested with pepsin and the intestinal peptides would be an effective alternative method.

In conclusion, four ACE inhibitory peptides were separated from the hydrolysate of boneless chicken leg meat digested with artificial gastric juice. Among these peptides, two peptides were identified as the peptides encrypted in myosin heavy chain. The

Table 4. ACE Inhibitory Peptides Derived from Porcine and Chicken Meats

source	sequence	parent protein	enzyme	IC_{50} (μM)	reference
	MNVKHWPWMK	myosin	pepsin	228	this work
	VTVNPYKWLP	myosin	pepsin	5.5	this work
	FQKPKR	myosin	thermolysin	14	*3*
	LKA	creatine kinase	thermolysin	8.5	*3*
	LKP	aldolase	thermolysin	0.32	*3*
chicken	LAP	musle	thermolysin	14	*3*
	IVGRPRHQG	actin	thermolysin	2.4	*3*
	FKGRYYP	creatine kinase	thermolysin	0.55	*3*
	IKW	muscle	thermolysin	0.21	*3*
	GFHypGLHypGP[a]	collagen	*Aspergillus* species-derived protease	42	*19*
	GAHypGLHypGP	collagen	*Aspergillus* species-derived protease	29	*26*
	ITTNP	myosin	thermolysin	549.0	*27*
	MNPPK	myosin	thermolysin	945.5	*27*
	MNP	myosin	synthesized	66.6	*27*
	NPP	myosin	synthesized	290.5	*27*
	ITT	myosin	synthesized	678.2	*27*
porcine	TTN	myosin	synthesized	672.4	*27*
	TNP	myosin	synthesized	207.4	*27*
	RMLGQTPTK	troponin C	pepsin	34	*28*
	RMLGQTP	troponin C	pepsin	503	*28*
	EKERERQ	troponin	pepsin	552.5	*18*
	KRQKYDI	troponin	pepsin	26.2	*18*

[a] Hyp = hydroxyproline.

peptide P1 (MNVKHWPWMK) corresponds to the amino acid sequence from amino acids 825 to 834 of myosin heavy chain, and the peptide P4 (VTVNPYKWLP) corresponds to the amino acid sequence from amino acids 125 to 135 of myosin heavy chain. They are novel ACE inhibitory peptides derived from chicken. Using synthetic peptides, IC_{50} values of P1 and P4 were determined as 228 and 5.5 μM, respectively. Because the peptide P4 has a relatively low IC_{50} value, it is a good starting substance for designing food supplements for hypertensive patients.

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