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Preparation and Characterization of Novel Bioactive Peptides Responsible for Angiotensin I-Converting Enzyme Inhibition from Wheat Germ

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Abstract: Reported is the preparation of wheat germ (WG) hydrolyzate with potent angiotensin I-converting enzyme (ACE) inhibitory activity, and the characterization of peptides responsible for ACE inhibition. Successful hydrolyzate with the most potent ACE inhibitory activity was obtained by 0.5 wt.%–8 h *Bacillus licheniformis* alkaline protease hydrolysis after 3.0 wt.%–3 h α-amylase treatment of defatted WG (IC_{50}; 0.37 mg protein ml^{-1}). The activity of WG hydrolyzate was markedly increased by ODS and subsequent AG50W purifications (IC_{50}; 0.018 mg protein ml^{-1}). As a result of isolations by high performance liquid chromatographies, 16 peptides with the IC_{50} value of less than 20 μM, composed of 2–7 amino acid residues were identified from the WG hydrolyzate. Judging from the high content (260 mg in 100 g of AG50W fraction) and powerful ACE inhibitory activity (IC_{50}; 0.48 μM), Ile-Val-Tyr was identified as a main contributor to the ACE inhibition of the hydrolyzate. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: ACE inhibitory peptide; hypertension; wheat germ hydrolyzate; alkaline protease

INTRODUCTION

Angiotensins (ANGs), in particular ANG II, III, and (1–7) are known to play important physiological roles as a pressor substance in the circulatory and/or localized renin–angiotensin systems. Therefore, pharmatherapeutic examinations to suppress the promotion of blood pressure in hypertensives have been attempted by inhibiting the production of these active ANGs or retarding the catalytic action of angiotensin I-converting enzyme (ACE, EC 3.4.15.1) [1]. As a point of this view, synthetic ACE inhibitors, such as captopril (IC_{50}; 21 nM) and enalapril (IC_{50}; 3 nM) have been developed and subjected to clinical use [2].

The study of the preparation and identification of natural ACE inhibitors derived from foodstuffs has become an active area of research to maintain our homeostasis by taking food [3–5]. Provided that the food has an *in vivo* exclusive depressor effect, the prophylaxis effect in hypertensives would be great. A large number of ACE inhibitory peptides have already been isolated from food proteins, such as casein [3], tuna muscle [4], zein [6], and so on [7–10], some of which were found to be effective for *in vivo* antihypertensive effect. Our recent observation on the ability of *Bacillus licheniformis* alkaline protease hydrolyzate of sardine muscle [7] to lower blood pressure (BP) of mild hypertensive subjects [11], suggest that appropriate enzymatic conversion

Abbreviations: ACE, angiotensin I-converting enzyme; WG, wheat germ; ANG, angiotensin; TNBS, 2,4,6-trinitrobenzene sulfonate; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; CH_3CN, acetonitrile; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; BP, blood pressure; SHR, spontaneously hypertensive rat.

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of food proteins to specific peptide may be efficient for inducing potent antihypertensive effect comparable with therapeutic drugs. Cheung *et al.* [12] demonstrated that the ACE inhibitory action by peptides depended on the affinity of *N*- and/or *C*-terminal amino acid residues for the ACE active site. From this point of view, we have isolated 12 ACE inhibitory peptides from sardine muscle hydrolyzate [13], and revealed that the peptide, Val-Tyr (VY) with the IC_{50} value of 5.2 μM, acted as a key ACE inhibitor [14].

The first aim of this study is to prepare physiological functional food materials from food protein with poor utility. In this report, wheat germ (WG) was selected as a source of food protein. Only one study concerning ACE inhibition by wheat gluten has been reported [15], in which Ile-Ile-Tyr was identified as an ACE inhibitor. However, there has been no attempt to study antihypertensive effect for WG, due to the difficulty in the release of protein from the matrix with high sugar (47%) and lipid (9.7%) contents. The second aim is to specify the prominent peptide responsible for ACE inhibition from the WG hydrolyzate.

MATERIALS AND METHODS

Materials and Reagents

WG was supplied from Taiyo Seifun Co. (Fukuoka, Japan). Enzymes used in this study were *Bacillus* α-amylase from Amano Seiyaku, *porcine gastric mucosa* pepsin, *bovine pancreas* chymotrypsin, and *bovine pancreas* trypsin from Boehringer Mannheim, *Bacillus licheniformis* alkaline protease from Novo (2.4L, TYPE FG), and *Aspergillus niger* protease YP-SS from Yakuruto Yakuhin Kogyo. Purified rabbit lung ACE was purchased from Sigma. ODS-AQ 120-S50 and AG50W-X8 resins were from YMC and Bio-Rad, respectively. Cosmosil 5C18-ARII and Cosmosil 5 Ph columns were from Nacalai Tesque. All other reagents used in this study were purchased from Nacalai Tesque.

Assay for ACE Inhibitory Activity

ACE inhibitory activity was determined by the proposed 2,4,6-trinitrobenzene sulfonate (TNBS) method [16]. For each assay, 25 μl of ACE inhibitor and 50 μl of 12.5 mM hippuryl-L-histidyl-L-leucine (Hip-His-Leu) as a substrate in a borate buffer (pH 8.3) containing 200 mM NaCl were incubated with 50 μl of ACE (25 mU ml^{-1}) at 37°C for 1 h. The reaction was stopped by adding 125 μl of 0.5 M HCl, the solution was adjusted to pH 9.12, and 25 μl of 0.1 M TNBS in a 0.1 M Na_2HPO_4 solution were then added. After incubating at 37°C for 20 min, 4.5 ml of 4 mM Na_2SO_3 in a 0.2 M NaH_2PO_4 solution were added, and the absorbance of the yielded TNP-His-Leu complex at 416 nm was measured with a Shimadzu UV-1200 spectrophotometer. The ACE inhibitor concentration required to inhibit 50% of the ACE activity under the assayed conditions was defined as the IC_{50} value. Total ACE inhibitory activity (unit) was defined as the quotient of the weight of inhibitor or hydrolyzate divided by its IC_{50} value.

Enzymatic Hydrolysis of Wheat Germ

Ten grams of intact or defatted WG by *n*-hexane at 80°C for 5 h was dissolved in 100 ml of deionized water, and homogenized with a Polytron for 2 min. The homogenate with or without α-amylase treatment was then subjected to enzymatic hydrolysis. The concentration of enzymes and incubation time examined in this study to give maximal ACE inhibition are discussed in the results and discussion section. Unless otherwise specified, incubation conditions were pH 3.0 at 40°C for pepsin and protease YP-SS, pH 8.0 at 40°C for chymotrypsin and trypsin, and pH 9.0 at 50°C for alkaline protease. After the hydrolysis, the solution was readjusted to pH 7.0 by adding 20% HCl or 20% NaOH and then heated for 15 min in a boiling water bath. After the centrifugation at 10000 rpm for 15 min, the supernatant was filtrated with Toyo filter paper (No. 1, Toyo Roshi).

ODS Separation of WG Hyrolyzate

One hundred milliliters of the alkaline protease hydrolyzate (A-1) was treated with 5 g of activated charcoal powder, and then applied on a YMC ODS-AQ 120-S50 column (3.5 × 13 cm) with stepwise elution of 500 ml of 0, 10, 25, 50 and 99.5% ethanol (each fraction was denoted as F-1, F-2, F-3, F-4 and F-5, respectively). Each fraction was collected and dried.

AG50W-X8 Separation of WG Hyrolyzate

Twenty milligrams of the most potent ACE inhibitory ODS fraction in 6 ml of deionized water were directly put on an AG50W-X8 cation exchange (H^+) disposable column, and after rinsing with 10 ml of water, it was eluted with 10 ml of 1.0 M

NH_4OH solution. The eluted fraction was lyophilized.

Purification of ACE Inhibitory Peptides by HPLC

ACE inhibitors were purified from the AG50W fraction by four-step HPLC (Shimadzu LC-9A instrument, Kyoto, Japan). In the first step, the fraction was applied on a Cosmosil 5 Ph column (4.6 × 250 mm) and eluted with a linear acetonitrile (CH_3CN) gradient (5–35%, 30 min) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.5 ml min^{-1}, while monitoring the absorbance at 220 nm. The fractions with ACE inhibitory activity were combined and concentrated, and then subjected to the second step HPLC (Cosmosil 5C18-ARII, 4.6 × 250 mm). The elution was done in the linear gradient mode of CH_3CN (5–35%, 60 min) at a flow rate of 0.5 ml min^{-1}. Next, the active fractions were purified on the same reversed phase column as the second step under the elution condition of CH_3CN (5–35%, 80 min) in 0.1% TFA at a flow rate of 0.4 ml min^{-1}. Final separation was done on the Cosmosil 5 Ph column under the elution condition of CH_3CN (5–26%, 37 min) at a flow rate of 0.3 ml min^{-1}.

Amino Acid Analysis

The amino acid composition was analyzed with a Shimadzu LC-6A amino acid analyzer after hydrolysis of 6 N HCl for 24 h at 110°C. The amino acid sequence was analyzed by automated Edman degradation using a Shimadzu PPSQ-21 protein sequencer. Identified peptide was synthesized by using Fmoc solid phase synthesis on a Kokusan Peptide Synthesizer (Kokusan Chemical Works Ltd., Tokyo, Japan).

Digestion Test

ACE inhibitor (1 mM) or 10 mg of WG preparations were incubated in 1 ml of pepsin, chymotrypsin or trypsin solution (0.03 mg ml^{-1}, pH 1.2, 0.1 M HCl or pH 6.8, 0.1 M phosphate buffer) for 4 h at 37°C individually. On successive digestion tests by chymotrypsin and trypsin after pepsin treatment, the pepsin solution was lyophilized, and then redissolved in 1 ml of the solution containing both chymotrypsin and trypsin (each concentration, 0.015 mg ml^{-1}), followed by incubation for 4 h at 37°C. The reaction was stopped by boiling for 10 min.

Determination of the Content of ACE Inhibitor

The determination of the content of ACE inhibitor in the WG hydrolyzate, ODS and AG50W-X8 fractions was done by the two-step HPLC. An aliquot (10 μl) of each sample (16 mg ml^{-1}) was injected into the Cosmosil 5 Ph column, and eluted with the linear gradient of CH_3CN (5–35%, 30 min) in 0.1% TFA at a flow rate of 0.5 ml min^{-1}. The fraction corresponding to the elution of the standard peptide was collected. The amount of the ACE inhibitor was determined with the second HPLC (Cosmosil 5C18-ARII) in the linear gradient mode of CH_3CN (5–9%, 30 min) in 0.1% TFA at a flow rate of 0.4 ml min^{-1}. Prior to the determination, calibration curve of ACE inhibitor was measured using their standards under the same HPLC conditions as described above. The content of ACE inhibitor in each sample was calculated by using this calibration curve. Each result is expressed as the mean (mg per 100 g sample) ± S.E. (%) of three independent experiments.

RESULTS AND DISCUSSION

Preparation of ACE Inhibitors from WG Protein by Enzymatic Hydrolysis

WG is known to have poor utility in the food industry in Japan, even though it is rich in vitamin E. To achieve a widespread usage of it, attempts to improve its functionality were primarily done in this study, especially with respect to the physiological function involved in blood pressure regulation. Table 1 summarizes the ACE inhibitory activities of WG hydrolyzates prepared by 1.0 wt.% addition of proteases for 5 h under various pretreatment conditions. α-Amylase treatment was done by adding 1.0 wt.% for 5 h at pH 7.0, 37°C. In all pretreatment conditions, ACE inhibitory activity varied with proteases, indicating that any peptides capable of inhibiting ACE were derived from WG protein. Among the proteases used in this study, hydrolysis of WG by alkaline protease was the most favorable in producing potent ACE inhibitors. Defatting of WG, followed by the treatment of α-amylase resulted in a ca. 1.1–2.5-fold higher potency of ACE inhibition than those in intact WG; the most potent ACE inhibition with the IC_{50} value of 0.67 mg protein ml^{-1} was obtained in alkaline protease hydrolyzate. This was thought to be due to easy susceptibility of WG protein to protease hydrolysis by removing lipids and sugars from the matrix. The

Table 1 ACE Inhibitory Activity of WG Hydrolyzates Prepared by Various Hydrolysis Conditions

Pretreatment[a]	Protease[b]	Yield (mg protein ml^{-1})	IC_{50} [c](mg protein ml^{-1})
Intact			
	Pepsin	22.2	2.38
	Chymotrypsin	24.8	2.68
	Trypsin	26.9	3.03
	Alkaline protease	29.0	1.69
	Protease YP-SS	14.7	2.56
Defatted			
	Pepsin	25.4	1.50
	Chymotrypsin	25.7	2.73
	Trypsin	31.4	2.93
	Alkaline protease	29.3	0.98
	Protease YP-SS	19.4	2.44
Defatted and α-amylase			
	Pepsin	27.3	1.78
	Chymotrypsin	26.3	2.40
	Trypsin	36.2	2.49
	Alkaline protease	29.2	0.67
	Protease YP-SS	20.9	1.77

[a] Pretreatment conditions of WG were as follows: defatting by *n*-hexane; 80°C for 5 h, α-amylase; 1.0 wt.% addition for 5 h at 37°C.
[b] Hydrolysis of WG by a given protease was done under the conditions of 1.0 wt.% addition for 5 h.
[c] ACE inhibitory activity was defined as an IC_{50} value.

efficacy of the defatting treatment was also supported by Sugiyama *et al.* [17], where the defatted sardine protein hydrolyzate by alkaline protease showed more powerful ACE inhibition with the IC_{50} value of 0.18 mg protein ml^{-1} than the intact one (IC_{50}; 0.26 mg protein ml^{-1} [7]).

The effect of concentration of α-amylase on ACE inhibitory activity was then examined in different fixed incubation times up to 24 h (Figure 1) under the WG hydrolysis condition of 1.0 wt.% alkaline protease at 50°C for 5 h. In each fixed incubation time, the inhibitory activity increased with the concentration of enzyme up to 5.0 wt.%. With 3.0 and 5.0 wt.% additions, the activity reached to the maximum with only 3 h of incubation. The IC_{50} value for both additions was 0.37 mg protein ml^{-1}, showing 1.8-fold higher activity than that of 1.0 wt.%–5 h treatment (IC_{50}; 0.67 mg protein ml^{-1}). Thus, the condition of 3.0 wt.% addition and 3 h of incubation was adequate for α-amylase treatment. Under this pretreatment condition, the hydrolysis conditions by alkaline protease to yield more potent ACE inhibitory activity were then investigated. Although data were not shown, the effects of the hydrolysis time (up to 24 h) and enzyme concentration (0.5–3.0 wt.%) on ACE inhibitory activity gave a similar behavior as in Figure 1. Namely, for all additions maximum activity was observed with only 8 h of hydrolysis time. In addition, little difference between the IC_{50} values of all additions (0.37 mg

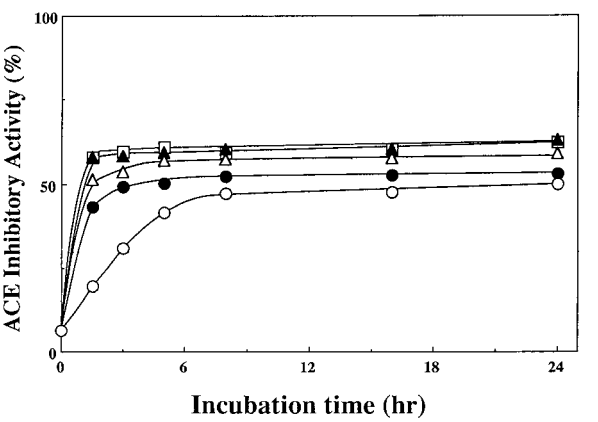

Figure 1 Effects of α-amylase treatment time on ACE inhibitory activity of alkaline protease WG hydrolyzate as a function of enzyme concentration. After defatted WG was treated by α-amylase at 37°C and pH 7.0, the 1.0 wt.%–5 h alkaline protease hydrolysis of it was done. The concentration of α-amylase examined in this assay was 0.5 wt.% (○), 1.0 wt.% (●), 2.0 wt.% (△), 3.0 wt.% (▲), 5.0 wt.% (□).

Table 2 ODS Separation of ACE Inhibitors from Wheat Germ Hydrolyzate[a]

Fraction	Protein g (yield)	IC_{50} (mg protein ml^{-1})
A-2[b]	12.4 (100)	0.15
F-1[c]	7.2 (58)	N.D.[d]
F-2	1.5 (12)	0.081
F-3	1.5 (12)	0.21
F-4	0.2 (2)	0.95
F-5	0 (0)	N.D.

[a] WG hydrolyzate (A-1) was obtained by 0.5 wt.%–5 h alkaline protease hydrolysis of defatted and 3.0 wt.%–3 h α-amylase treated WG.
[b] The A-1 was treated with active charcoal powder, and the eluted fraction (A-2) was applied on a YMC ODS-AQ12OT column.
[c] Successive elution with 500 ml of 0, 10, 25, 50 and 99.5% ethanol (denoted as F-1 to F-5, respectively) was done.
[d] Not detected.

protein ml^{-1}) was observed. Consequently, 3.0 wt.%–3 h α amylase treatment of defatted WG, and the subsequent 0.5 wt.%–8 h alkaline protease hydrolysis was regarded as the best conditions in providing ACE inhibitory power to WG, although the activity was still weaker than that of the sardine muscle hydrolyzate (IC_{50}; 0.26 mg protein ml^{-1} [7]). In this study, 3.0 wt.% α-amylase or 0.5 wt.% alkaline protease solution did not inhibit ACE at all.

ODS Purification

In order to improve the ACE inhibitory potency of WG hydrolyzate (denoted as A-1) under the foregoing conditions, the fractionation on a YMC ODS-AQ120-S50 was then performed. Prior to applying the A-1 to the ODS column chromatography, the A-1 was treated with active charcoal powder to remove any browning substrates produced by the Maillard reaction. As a result, the eluted fraction (A-2) strongly retarded the action of ACE ([IC_{50}; 0.15 mg protein ml^{-1}). Then, the A-2 was subjected to the ODS column chromatography with successive elution of ethanol. As summarized in Table 2, the most potent ACE inhibition was observed in the 10% ethanol eluted fraction, F-2, showing the IC_{50} value of 0.081 mg protein ml^{-1}. As suggested in our previous report [7], the higher polar elution fraction of F-2 was useful in preparing physiologically functional food owing to good taste with little bitterness and high solubility in water. However, the ACE inhibitory activity of F-2 was still much lower than that of 10% ethanol eluted fraction of sardine muscle hydrolyzate (IC_{50}; 0.015 mg protein ml^{-1}). As we have already revealed, oral administration of this fraction of sardine muscle hydrolyzate (4 g day^{-1}) significantly lowered the BP of mild hypertensive subjects ($n = 17$: systolic/diastolic BP; -9.3 ± 2.0 mmHg/-5.2 ± 1.2 mmHg, 4 weeks after the protocol) [11]. Therefore, further improvement of the activity of F-2 would be required to achieve *in vivo* antihypertensive effect.

Cation Exchange Purification

The first attempt for the purification of F-2 was done by using 1 K Macrosep centrifugal concentrator ($M_W < 1000$; Filtron, USA) at $5000 \times g$ for 2 h. As a result, high activity with the IC_{50} value of 0.023 mg protein ml^{-1} was secured, while the yield of the fraction was only 3.6% of A-2 hydrolyzate. The alternative purification on an AG50W X-8 cation exchange column with 1.0 M NH_4OH resulted in excellent ACE inhibition (IC_{50}; 0.018 mg protein ml^{-1}) and good yield (8%), suggesting that this fractionation was the best procedure for preparing a bioactive food material in this study.

Isolation and Identification of ACE Inhibitors

For the aim of clarifying peptides responsible for ACE inhibition in WG hydrolyzate, isolation of inhibitory peptides in AG50W X-8 fraction was performed. Figure 2 shows the elution pattern of the AG50W X-8 fraction by Cosmosil 5 Ph column with the relative ACE inhibitory activity. Most of the potent ACE inhibitory activity occurred in two frac-

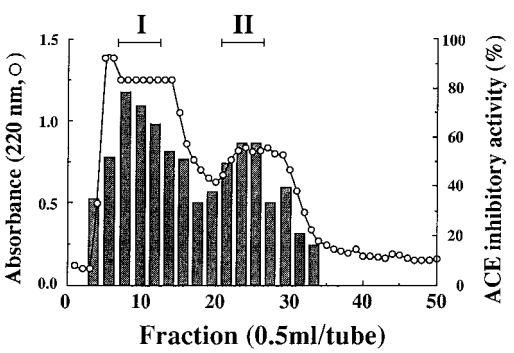

Figure 2 Elution profile of AG50W fraction of WG hydrolyzate eluted with 1.0 M NH_4OH from a Cosmosil 5 Ph column with a gradient of increasing CH_3CN (5–35%, 30 min) in 0.1% TFA at a flow rate of 0.5 ml min^{-1}. The fractions (3.5–6.5 ml and 11–14 ml) with ACE inhibitory activity were combined and depicted as I and II, respectively.

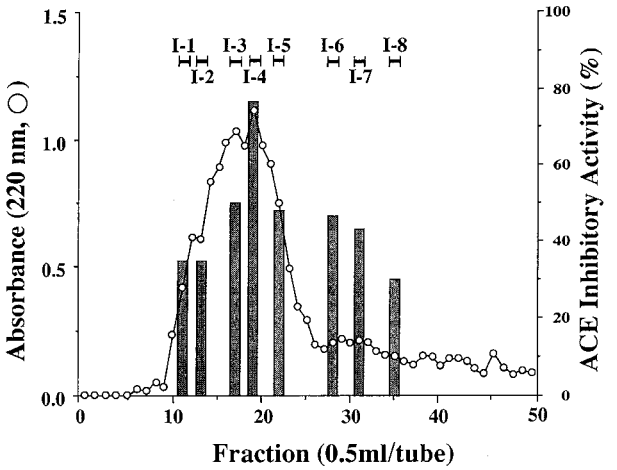

Figure 3 Separation of active fraction I eluted from Cosmosil 5 Ph column on Cosmosil 5C18-ARII column. The elution was done in the linear gradient mode of CH_3CN (5–35%, 60 min) at a flow rate of 0.5 ml min^{-1}. Each fraction with the activity was collected individually.

tions depicted as I and II (elution volume of 3.5–6.5 ml and 11–14 ml, respectively), though other fractions also showed minor inhibition. Both fractions were then applied to the second step of HPLC with Cosmosil 5C18-ARII column (Figure 3). Eight peaks (denoted I-1 to I-8) from fraction I, and six peaks (II-1 to II-6) from fraction II showed the ACE inhibition. Each active peak was again purified by the same column as mentioned above, and when the complete separation was not achieved, subsequent purification on Cosmosil 5 Ph column was done. Figure 4 shows the example of HPLC purification of active peak of I-1. The I-1 peak was separated into two peaks of I-1a and I-1b on Cosmosil 5C18-ARII column, and finally two peaks denoted as I-1a1 and I-1a2 were purified from I-1a on Cosmosil 5 Ph column. Consequently, 14 peaks were isolated from fraction I, nine peaks from fraction II. As a result of amino acid and sequence analyses, 16 peaks (ten peaks from fraction I and six peaks from fraction II) were identified as an ACE inhibitory peptide. The other seven isolated peaks unfortunately could not be identified due to their low yield. The retention time of each identified peptide on the final HPLC was as follows: Thr-Phe from I-1a1; 21.4 min, Leu-Tyr from I-1a2; 25.7 min, Tyr-Leu from I-1b1; 30.8 min, Ala-Phe from I-2a1; 20.1 min, Ile-Tyr from I-3a1; 19.1 min, Thr-Ala-Pro-Tyr from I-5a1; 28.2 min, Val-Phe from I-6a1; 34.3 min, Thr-Val-Val-Pro-Gly from I-7a1; 39.0 min, Asp-Tyr-Val-Gly-Asn from I-8a1; 9.4 min, Thr-Tyr-Leu-Gly-Ser from I-8e1; 40.6 min, Ala-Pro-Gly-Ala-Gly-Val-Tyr from II-1a1; 48.8 min, Thr-Val-Pro-Tyr from II-1b1; 51.3 min, Ile-Val-Tyr from II-2b1; 43.7 min, Gly-GlyVal-Ile-Pro-Asn from II-4a1; 45.8 min, Val-Phe-Pro-Ser from II-4a2; 47.8 min, Asp-Ile-Gly-Tyr-Tyr from II-6a1; 52.0 min.

Table 3 summarizes the identified ACE inhibitory peptides from the alkaline protease WG hydrolyzate with the ACE inhibitory activity of natural peptide. Twelve inhibitory peptides, except for Leu-Tyr, Tyr-

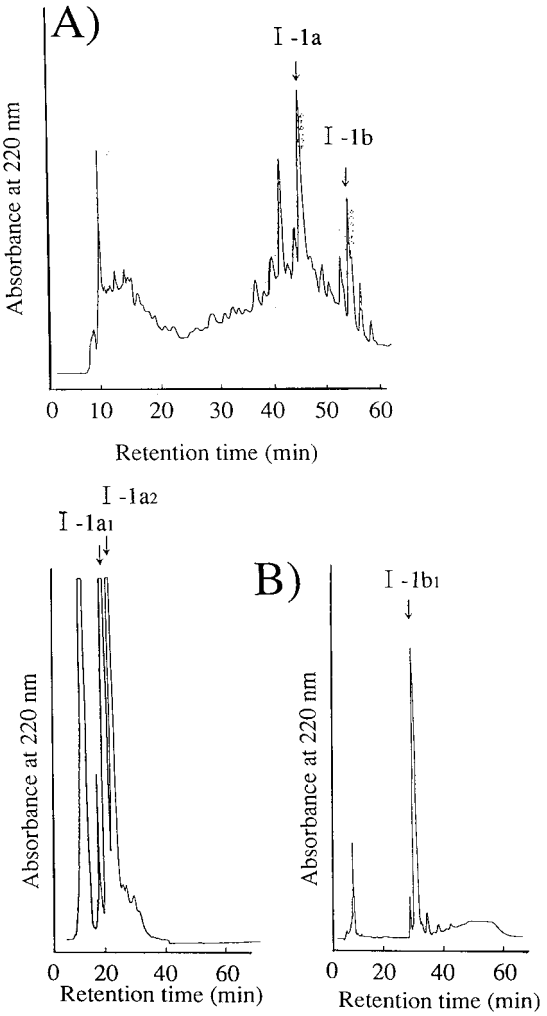

Figure 4 Purification of active peak of I-1 from Cosmosil 5C18-ARII column. The I-1 peak was separated into two peaks of I-1a and I-1b on Cosmosil 5C18-ARII column (A), and finally two peaks denoted as I-1al and I-1a2 were purified from I-1a on Cosmosil 5 Ph column (B). The elution on Cosmosil 5C18-ARII column was done in the linear gradient mode of CH_3CN (5–35%, 80 min) in 0.1% TFA at a flow rate of 0.4 ml min^{-1}. Final separation was achieved on the Cosmosil 5 Ph column under the elution condition of CH_3CN (5–26%, 37 min) at a flow rate of 0.3 ml min^{-1}.

Table 3 Amino Acid Sequence and IC_{50} Value of Natural ACE Inhibitors Isolated from Wheat Germ Hydrolyzate

Amino acid sequence	Amino acid ratio in acid hydrolyzate	IC_{50} (μM)
Thr-Phe	Thr 1.00, Phe 1.12	17.8
Leu-Tyr[a]	Leu 1.10, Tyr 1.00	6.4
Tyr-Leu[a]	Tyr 1.02, Leu 1.10	16.4
Ala-Phe	Ala 1.00, Phe 1.38	15.2
Ile-Tyr[b]	Ile 1.05, Tyr 1.00	2.1
Val-Phe[a,c]	Val 1.03, Phe 1.00	9.2
Ile-Val-Tyr	Ile 1.00, Val 1.02, Tyr 1.69	0.48
Val-Phe-Pro-Ser	Val 1.05, Phe 1.00, Pro 1.23, Ser 1.06	0.46
Thr-Ala-Pro-Tyr	Thr 1.00, Ala 1.28, Pro 1.17, Tyr 1.00	13.6
Thr-Val-Pro-Tyr	Thr 1.00, Val 1.55, Pro 1.83, Tyr 1.12	2.0
Thr-Val-Val-Pro-Gly	Thr 1.00, Val 1.84, Pro 1.10, Gly 1.36	2.2
Asp-Ile-Gly-Tyr-Tyr	Asp —, Ile 1.00, Gly 1.12, Tyr 1.56	3.4
Asp-Tyr-Val-Gly-Asn	Asp —, Tyr 1.00, Val 1.33, Gly 1.06, Asn —	0.72
Thr-Tyr-Leu-Gly-Ser	Thr 1.17, Tyr 1.00, Leu 1.30, Gly 1.57, Ser 1.08	0.86
Gly-Gly-Val-Ile-Pro-Asn	Gly 3.17, Val 1.03, Ile 1.00, Pro 1.73, Asn —	0.74
Ala-Pro-Gly-Ala-Gly-Val-Tyr	Ala 1.93, Pro 1.15, Gly 1.94, Val 1.01, Tyr 1.00	1.7

[a] [13]; [b] [8]; [c] [12].

Leu, Ile-Tyr and Val-Phe, which have already been reported by us [13] and other investigators [8,12], were found for the first time in natural protein hydrolyzate. All of these peptides had potent inhibitory activity with the IC_{50} values of < 20 μM. In particular, the peptides constructed of more than four amino acid residues showed the powerful ACE inhibitory activity of less than 5 μM, except for Thr-Ala-Pro-Tyr. However, by considering poor resistance of longer peptides to digestion by gastrointestinal proteases and low susceptibility to absorption [18], the isolated peptides with more than four amino acids might be of little significance toward the *in vivo* antihypertensive effect regardless of their high *in vitro* activity. Among the isolated peptides, Ile-Val-Tyr (IVY) was a unique competitive ACE inhibitor having the most powerful ACE inhibition of 0.48 μM (synthetic peptide; 0.58 μM) and the sequence of ACE inhibitory VY. As far as we know, this tripeptide was one of the strongest ACE inhibitors as well as Leu-Arg-Pro (IC_{50} of 0.27 μM) [19] in natural protein hydrolyzate. In addition, taking into consideration that the fragment peptide, VY, showed a significant *in vivo* antihypertensive effect in spontaneously hypertensive rat [20,21], IVY may be one of the noteworthy ACE inhibitory peptides in the WG hydrolyzate.

Content of ACE Inhibitor in the WG Preparations

In order to clarify the importance of IVY in the WG hydrolyzate, the content and the total ACE inhibitory activity of IVY in the WG hydrolyzate with the total ACE inhibitory activity of 13.5 units, ODS preparation eluted with 10% ethanol (61.7 units), and AG50W X-8 preparation eluted with 1.0 M NH_4OH (277.8 units) were then investigated (Table 4). As a result, 21.6, 143 and 258 mg of IVY were present in 100 g of WG hydrolyzate, ODS preparation and AG50W preparation, respectively. The magnitude of the ACE inhibitory contribution of IVY to the overall inhibition of each preparation was estimated to be 85, 12.3 and 4.9%, respectively. These results strongly suggested that IVY was one of the most prominent ACE inhibitory peptides in the WG hydrolyzate.

Table 4 Contents of Ile-Val-Tyr in WG Hydrolyzates with Total ACE Inhibitory Activity

	Content[a] (mg 100-g sample^{-1})	Total activity[b] (units)
WG	21.6 ± 3.3	1.1
ODS	143 ± 16.7	7.6
AG50W	258 ± 15.1	13.6

[a] The content of Ile-Val-Tyr in 100 g of WG hydrolyzate, ODS fraction eluted with 10% ethanol, and AG50W fraction eluted with 1.0 M NH_4OH were determined by the HPLC analysis. Data represent as the mean ($n = 3$) ± S.E. (%).

[b] Total ACE inhibitory activity (unit) was calculated by dividing the weight (g) of peptide in 1 g of each hydrolyzate by its IC_{50} value (g l^{-1}).

Table 5 Digestive Stability of WG Hydrolyzates and Ile-Val-Tyr Toward ACE Inhibition

Digestion[a]	IC_{50} (μg ml^{-1})			
	WG	ODS	AG50W	IVY
None	1450	120	19	0.19
Pepsin	1600	120	27	0.19
Chymotrypsin	1700	230	49	0.19
Trypsin	960	130	25	0.19
Pepsin → chymotrypsin and trypsin[b]	1050	165	31	0.19

[a] Ten milligrams of WG hydrolyzates or 1.0 mM of Ile-Val-Tyr was incubated in 1 ml of each protease solution (30 μg ml^{-1}) for 4 h at 37°C. Digestion stability was evaluated by comparing the IC_{50} value of digested hydrolyzates with that of untreated ones.
[b] The concentration of chymotrypsin and trypsin was set at 15 μg ml^{-1}.

Digestion Stability of ACE Inhibitor

Table 5 summarizes the change in IC_{50} values of WG preparations and IVY before and after treatment with gastrointestinal proteases to simulate in vivo resistance to digestion. As a result, the activity of WG hydrolyzate treated with pepsin and the subsequent chymotrypsin and trypsin showed 27% increase, indicating that any active peptides must be newly produced by action of these proteases, in particular trypsin. On the other hand, ODS and AG50W preparations lost the activity after the digestion. This was assumed to be caused by the enzymatic degradation or inactivation of longer bioactive peptides occurred in them (Table 3). The report by Saito *et al.* [22] has provided the evidence that tetra- or pentapeptides, such as Tyr-Gly-Gly-Tyr or Ile-Tyr-Pro-Arg-Tyr, were liable to the protease hydrolysis to form amino acids or dipeptides, whereas di- and tripeptides were highly resistant to digestion. Our result that IVY had no change in the activity after the digestion also supported the efficacy of smaller peptides on digestion stability.

CONCLUSION

The present *in vitro* study demonstrated that IVY was identified as a main contributor to *in vitro* ACE inhibition of the WG hydrolyzate. *In vivo* antihypertensive effects of WG hydrolyzate and IVY on SHR are now under investigation on the basis of these findings.

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REFERENCES

1. C.I. Johnston and L.M. Burrell (1995). Evolution of blockade of the renin–angiotensin system. *J. Hum. Hypertens. 9*, 375–380.
2. N.M. Hooper (1991). Angiotensin converting enzyme: implications from molecular biology for its physiological functions. *Int. J. Biochem. 23*, 641–647.
3. S. Maruyama and H. Suzuki (1982). A peptide inhibitor of angiotensin I-converting enzyme in the tryptic hydrolysate of casein. *Agric. Biol. Chem. 46*, 1393–1394.
4. S. Maruyama, K. Nakagomi, N. Tomizuka and H. Suzuki (1985). Angiotensin I-converting enzyme inhibitor derived from an enzymatic hydrolysate of casein: isolation and bradykinin-potensiating activity on the uterus and the ileum of rats. *Agric. Biol. Chem. 49*, 1405–1409.
5. Y. Kohama, S. Matsumoto, H. Oka, T. Teramoto, M. Okabe and T. Mimura (1988). Isolation of angiotensin converting enzyme inhibitor from tuna muscle. *Biochem. Biophys. Res. Commun. 155*, 332–337.
6. S. Miyoshi, T. Kaneko, Y. Yoshizawa, F. Fukui, H. Tanaka and S. Marnyama (1991). Hypotensive activity of enzymatic α-zein hydrolyzate. *Agric. Biol. Chem. 55*, 1407–1408.
7. T. Matsui, H. Matsufuji, E. Seki, K. Osajima, M. Nakashima and Y. Osajima (1993). Inhibition of angiotensin I-converting enzyme by *Bacillus licheniformis* alkaline protease hydrolyzates derived from sardine muscle. *Biosci. Biotech. Biochem. 57*, 922–925.
8. K Yokoyama, H. Chiba and M. Yoshikawa (1992). Inhibitors for angiotensin I-converting enzyme from thermolysin digest of dried bonito. *Biosci. Biotech. Biochem. 56*, 1541–1545.
9. K. Kimoto, Y. Kuroda, Y. Saito, J. Yamamoto, T. Murakami and Y. Aoyagi (1998). Purification and identification of angiotensin I-converting enzyme inhibitor from Morokheiya (*Corchorus olitorius*). *Food Sci. Technol. Int. Tokyo 4*, 223–226.
10. M.M. Mullally, H. Meisel and R.J. FitzGerald (1997). Identification of a novel angiotensin I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine β-lactoglobulin. *FEBS Lett. 402*, 99–101.
11. T. Kawasaki, K. Osajima, E. Seki, M. Yoshida, K. Asada, T. Matsui and Y. Osajima (1998). Antihypertensive effect of Val-Tyr, a short chain peptide derived from sardine muscle hydrolyzate, on hypertensives. *J. Hypertension 16(Suppl. 2)*, S25.

12. H.-S. Cheung, F.-L. Wang, M.A. Ondetti, E.F. Sabo and D.W. Cushman (1980). Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. *J. Biol. Chem. 255*, 401–407.
13. H. Matsufuji, T. Matsui, E. Seki, K. Osajima, M. Nakashima and Y. Osajima (1994). Angiotensin I-converting enzyme inhibitory peptides in an alkaline protease hydrolyzate derived from sardine muscle. *Biosci. Biotech. Biochem. 58*, 2244–2245.
14. T. Matsui, Y. Osajima, K Uezono and T. Kawasaki (1998). Metabolism of endogenous Val-Tyr with antihypertensive effect in human plasma. *J. Hypertension 16(Suppl. 2)*, S138.
15. Y. Kawamura (1991). Prospects and basic problems of physiologically active peptides derived from food proteins. *Shokuhin to Kaihatsu (in Japanese) 26*, 28–32.
16. T. Matsui, H. Matsufuji and Y. Osajima (1992). Colorimetric measurement of Angiotensin I-converting enzyme inhibitory activity with trinitrobenzene sulfonate. *Biosci. Biotech. Biochem. 56*, 517–518.
17. K. Sugiyama, K. Takada, M. Egaqa, I. Yamamoto, H. Onzuka and K. Oba (1991). Hypotensive effect of fish protein hydrolysate. *Nippon Nogeikagaku Kaishi (in Japanese) 65*, 33–43.
18. H. Hagihira and Y. Nakabou (1990). Absorption and metabolism of peptides. *Taisha (in Japanese) 27*, 993–1000.
19. S. Miyoshi, H. Ishikawa, T. Kaneko, F. Fukui, H. Tanaka and S. Maruyama (1991). Structure and activity of angiotensin-converting enzyme inhibitors in an α-zein hydrolysate. *Agric. Biol. Chem. 55*, 1313–1318.
20. H. Matsufuji, T. Matsui, S. Ohshige, T. Kawasaki, K Osajima and Y. Osajima (1995). Antihypertensive effects of angiotensin fragments in SHR. *Biosci. Biotech. Biochem. 59*, 1398–1401.
21. Y. Saito, K Wanezaki, A. Kawano and S. Imayasu (1994). Antihypertensive effects of peptide in Sake and its by-products on spontaneously hypertensive rats. *Biosci. Biotech. Biochem. 58*, 812–816.
22. Y. Saito, K Wanezaki, A. Kawano and S. Imayasu (1994). Structure and activity of angiotensin I converting enzyme inhibitory peptides from Sake and Sake lees. *Biosci. Biotech. Biochem. 58*, 1767–1771.

