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Latent Production of Angiotensin I-Converting Enzyme Inhibitors from Buckwheat Protein

CHUN-HUI LI,[a] TOSHIRO MATSUI,[a]* KIYOSHI MATSUMOTO,[a] RIKIO YAMASAKI[b] and TERUKAZU KAWASAKI[c]

[a] Faculty of Agriculture, Division of Bioresource and Bioenvironmental Sciences, Graduate School Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan
[b] Matsumoto Factory, Nikkoku Flour Mills Co. Ltd, 2-6-52 Minamimatsumoto, Matsumoto 390-0832, Japan
[c] Institute of Health and Sport Science Kyushu Sangyo University, 2-3-1 Matsukadai, Higashi-ku, Fukuoka 813-8503, Japan

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Abstract: The latent production of angiotensin I-converting enzyme (ACE) inhibitors from tartary buckwheat (BW) was investigated, and the peptides responsible for ACE inhibition characterized. Intact buckwheat was found to exhibit ACE inhibitory activity having an IC_{50} value of 3.0 mg/ml. The activity of the protein fraction (IC_{50}: 0.36 mg protein/ml) was not enhanced by pepsin treatment. Pepsin, followed by chymotrypsin and trypsin hydrolysis, resulted in a significant increase in the ACE inhibitory activity (IC_{50}: 0.14 mg protein/ml). The rutin contained in the buckwheat did not exhibit any ACE inhibition. A single oral administration of BW digest lowered the systolic blood pressure of a spontaneously hypertensive rat. Thus, BW proteins offer a potential resource for producing ACE inhibitory peptides during the digestion process. From the di-/tri-peptide fraction (DTPF) of the BW digest, inhibitory peptides were identified. The magnitude (%) of the total ACE inhibitory contribution of each identified peptide, relative to the overall inhibition of the DTPF, was about 41%.

Keywords: ACE inhibitory peptide; hypertension; buckwheat digest; gastrointestinal protease

INTRODUCTION

A clinical examination made in the Mustang District of Nepal, where the inhabitants rely on buckwheat (BW) as their principal food, demonstrated that the pathogenesis of hypertension was quite low (at less than 25% in the Thakali Tribe) regardless of their salt tea intake [1].

It is well known that buckwheat is an edible and rich source of rutin, a flavonol glycoside compound (quercetin-3β-D-rutinoside) [2]. Tartary buckwheat seed, for example, has a high rutin content of about 1.5% [3]. In addition, it has been reported that rutin is effective in improving the capillary fragility responsible for maintaining normal blood pressure (BP) [4]. However, there has been no evidence as to whether rutin is directly involved in lowering blood pressure. Iwata *et al.* [5] demonstrated that the long-term feeding of Kangra buckwheat to spontaneously hypertensive rat (SHR) resulted in a significant blood pressure reduction of 14%, as well as a reduction in the plasma triglyceride level after 4–6 weeks. Wheat flour, which was being used as a control diet, did

Abbreviations: ACE, angiotensin I-converting enzyme; AcOEt, ethylacetate; BP, blood pressure; BW, buckwheat; CH_3CN, acetonitrile; DTPF, di-/tri- peptide fraction; HPLC, high-performance liquid chromatography; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat; TFA, trifluoroacetic acid; TNBS, 2,4,6-trinitrobenzene sulphonate.

* Correspondence to: Dr Toshiro Matsui, Institute of Food Biotechnology, Division of Bioresource and Bioenvironmental Sciences, Graduate School of Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan; e-mail: tmatsui@agr.kyushu-u.ac.jp
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not. This prompted us to further investigate whether any of the active constituents present in buckwheat could be associated with blood pressure reduction in SHR.

Our studies [6–8] revealed the efficacy of natural angiotensin I-converting enzyme (ACE, EC 3.4.15.1) inhibitors and protein hydrolysates in reducing blood pressure. Almost all the natural hypotensive ACE inhibitors are peptides, notably di-/tripeptides, because of their high competitive affinity with the ACE active site. Based on the concept proposed by Cheung *et al.* [9], many natural ACE inhibitory peptides in sardine muscle [10] and wheat germ hydrolysates [7] were successfully identified. SHR [11] and human studies [12] involving the oral administration of sardine muscle hydrolysate also revealed the hypotensive ability of bioactive peptides through their retardation of ACE activity.

In this report, therefore, the function of buckwheat constituents with respect to blood pressure regulation through ACE inhibition was examined.

MATERIALS AND METHODS

Materials and Reagents

Tartary buckwheat flour was supplied by Nikkoku Flour Mill (Nagano, Japan). The enzymes used were porcine gastric mucosa pepsin, bovine pancreas chymotrypsin and bovine pancreas trypsin, all of which were supplied by Boehringer Mannheim (Penzberg, Germany). Purified rabbit lung ACE was purchased from Sigma (St Louis, MO). The Cosmosil 5C18-ARII and Cosmosil 5Ph columns were supplied by Nacalai Tesque (Kyoto, Japan). The amino acid standards were purchased from Wako Pure Chemical Industries (Osaka, Japan), while the peptide standards came from the Peptide Institute (Osaka, Japan). All other reagents used in this study were purchased from Nacalai Tesque. These reagents were used as supplied without any further purification. The HPLC-grade water for all applications was produced using a Milli-Q purification system manufactured by Millipore (Bedford, MA).

Assay for ACE Inhibitory Activity

The ACE inhibitory activity was determined by applying a modified Lieberman's method [13]. A mixture (150 μl) containing 50 μl of ACE inhibitor and 100 μl of ACE (25 mU/ml) was incubated for 5 min at 37 °C. Then, the reaction was initiated by adding 100 μl of 12.5 mM hippuryl-L-histidyl-L-leucine as a substrate in a borate buffer (pH 8.3) containing 1 M NaCl. After subsequently stopping the reaction by adding 250 μl of 0.5 M HCl, the hippuric acid was extracted with 1.5 ml of ethylacetate (AcOEt). An aliquot (500 μl) from the AcOEt layer was evaporated to dryness and then dissolved in 3 ml of 1 M NaCl. The concentration of the resulting hippuric acid was determined from its absorbance at 228 nm using a Shimadzu UV-1200 spectrophotometer. Thus, the concentration of ACE inhibitor required to inhibit 50% of the ACE activity under these assayed conditions was defined as the IC_{50} value. The protein content (nitrogen × 6.25) was analyzed by means of element analysis (Center of Elementary Analysis, Kyushu University) and, as a result, the total ACE inhibitory activity (unit) was defined as the quotient of the weight of the inhibitor or digest divided by its IC_{50} value.

Preparation of Buckwheat Digest

Ten grams of heat-treated (98 °C, 10 min) ground BW powder was dissolved in 100 ml of deionized water, and then the solution was homogenized in a Polytron for 2 min. The homogenate was subjected to protease hydrolyses with pepsin (pH 1.2, 4 h, 37 °C), followed by chymotrypsin and trypsin (pH 6.8, 4 h, 37 °C). The enzyme : substrate ratio was 1 : 100 (w/w). The homogenate was heated to 98 °C for 10 min to deactivate the enzymes, centrifuged at 8500 g for 15 min, and then the supernatant was filtered and lyophilized.

Measurement of Antihypertensive Effect in SHR

Ten male SHRs (aged 15 weeks, each weighing 324.7±9.8 g) were purchased from Charles River Japan, Inc., (Yokohama, Japan). They were fed a basal diet (CE-2, Clea Japan, Tokyo) and tap water *ad libitum*. The animals were housed together in a single cage with a cycle of 12 h lights-on/12 h lights-off in a temperature-controlled room at 22 °C for 2 weeks. Those SHRs with a systolic blood pressure (SBP) in excess of 180 mmHg were used for this study. The SHRs were divided into two groups, only one of which was orally fed a diet supplemented with the BW digest. After a single oral administration, the SBP was measured after 2, 4, 6 and 8 h. A tail-cuff (model BP-98A, Softron, Tokyo, Japan) was used to take three successive SBP measurements for each conscious rat after

keeping the rat at 39.5 °C for 10 min. The mean of the three measurements was taken as the BP value.

Gel-permeation Purification of Buckwheat Digest

The di- and tri-peptide fraction (DTPF) of the BW digest was obtained by gel-permeation chromatography with a Superdex Peptide HR 10/30 column (1.0 × 30 cm; Pharmacia Biotech AB, Uppsala, Sweden) [14]. It was eluted using 30% acetonitrile (CH_3CN) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.3 ml/min with detection at 220 nm (35 °C). The following molecular size markers were used: Angiotensin II (M_w 1,146), Gln-Val-Lys (M_w 373), Gly-Tyr (M_w 238) and Gly-Gly (M_w 132). An aliquot (100 µl) of the digest (10 mg/ml) was injected into the HPLC (Shimadzu LC-6A, Kyoto, Japan), and then the DTPF was obtained using a calibration curve of the logarithmic molecular weight against elution time, in which the fraction between 52 min (corresponding to the elution time of Trp-Trp-Trp) and 69 min (that of Gly-Gly) was collected.

Estimation of DTPF Average Peptide Length

An aliquot (25 µl) of the desired digest (6 mg/ml), either before or after acid hydrolysis, was added to 25 µl of 0.1 M 2,4,6-trinitrobenzene sulphonate (TNBS) in a 0.1 M Na_2HPO_4 solution, and then the mixture was incubated at 37 °C for 20 min. Acid hydrolysis with 6 M HCl containing 1% phenol at 150 °C was performed for 1 h. After adding 1 ml of 4 mM Na_2SO_3 to a 0.2 M NaH_2PO_4 solution, the trinitrophenyl-NH complex was measured at 416 nm with a Shimadzu UV-1200 spectrophotometer. The average peptide length of the DTPF was estimated by calculating the ratio of absorbance of the free amino groups with TNBS after hydrolysis (A_a) relative to that prior to hydrolysis (A_b), i.e. average peptide length = A_a/A_b. Each analysis was performed twice.

Isolation of Peptides in DTPF of Buckwheat Digest by HPLCs

The ACE inhibitory peptides in the DTPF of the BW digest were isolated by applying the multi-step process of reversed-phase HPLC (Shimadzu LC-9A, Kyoto, Japan). In the first step, 10 µl of DTPF (120 mg/ml) was applied in a Cosmosil 5Ph column (4.6 × 250 mm) and then eluted with a linear gradient mode of CH_3CN (5%–35%, 30 min) containing 0.1% TFA at a flow rate of 0.3 ml/min, while the absorbance was monitored at 220 nm. Each fraction with ACE inhibitory activity was collected individually and then the final step of HPLC (Cosmosil 5C18-ARII, 4.6 × 250 mm) was performed with elution by 5% CH_3CN containing 0.1% TFA or a linear gradient mode of CH_3CN (5%–35%, 60 min) containing 0.1% TFA at a flow rate of 0.3 ml/min.

Amino Acid Analysis

The amino acid composition was analysed with an HPLC system consisting of a Shimadzu LC-10A, a Shimadzu CTO-10A column oven, a Shimadzu RF-10AXL fluorescence detector and a Waters AccQ · Tag column (0.39 × 15 cm, Milford, MA). The amino acid sequence was analysed by applying automated Edman degradation using a Shimadzu PPSQ-21 protein sequencer, coupled with the HPLC identification of the resulting phenylthiohydantoin amino acids.

RESULTS AND DISCUSSION

ACE Inhibitory Activity of Buckwheat Digest and Rutin

In some areas of the world, buckwheat constitutes an essential source of dietary proteins such as globulin and albumin, as well as flavonols (rutin or quercetin). Tartary buckwheat has been shown to contain two kinds of globulin with molecular weights of 443 kDa and 679 kDa [15]. Only one study [5] on the antihypertensive effect of buckwheat intake reported that it significantly lowered blood pressure by 30 mmHg in SHR after 4–6 weeks of incorporating it into the diet. Despite this finding, however, there was no evidence as to which constituents of buckwheat were responsible for reducing blood pressure.

To confirm whether buckwheat has an ACE inhibitory effect, digestion was examined using the gastrointestinal proteases of tartary buckwheat flour. Table 1 summarizes the changes in the IC_{50} values of tartary buckwheat flour preparations both before and after *in vitro* gastrointestinal digestion. The peptic hydrolysis of buckwheat exhibited ACE inhibitory activity (IC_{50}: 0.35 mg protein/ml) that was similar to that of intact buckwheat (IC_{50}: 0.36 mg protein/ml), suggesting that pepsin treatment is not effective in eliciting the ACE inhibitory

Table 1 Change in ACE Inhibitory Activity of Tartary Buckwheat Flour after Gastrointestinal Digestion[a]

Digestion	IC_{50} (mg/ml)	IC_{50} (mg protein/ml)	Yield (mg)	Yield[b] (mg protein)
None	3.0	0.36	1000	121
Pepsin (1.0 wt%, 4 h)	1.2	0.35	197	58
Pepsin (1.0 wt%, 4 h) ⟶ chymotrypsin + trypsin (each 0.5 wt%, 4 h)	0.30	0.14	127	62

[a] Gastrointestinal digestion was done with pepsin (pH 1.2), and then with chymotrypsin and trypsin (pH 6.8) at 37 °C. Tartary buckwheat flour with heat treatment (98 °C, 10 min) was used for digestion.
[b] Protein content (nitrogen × 6.25) was analysed by element analysis (Center of Elementary Analysis, Kyushu University).

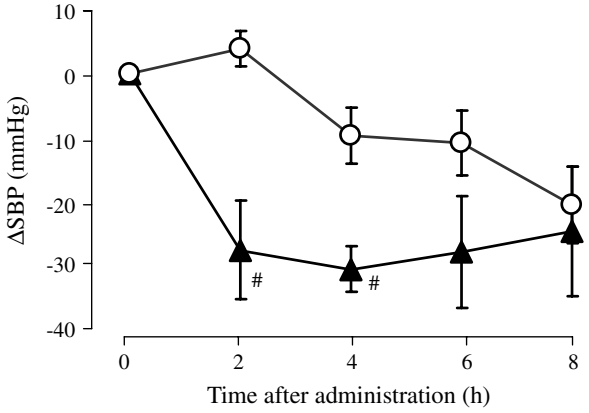

Figure 1 Effect of single oral administration of buckwheat digest on systolic blood pressure of 17-week SHR. Changes in SBP from zero time are expressed as mean ± SEM ($n = 5$). Treatments were control (○), 100 mg/kg of body weight (▲). # Significantly different from control group at $p < \leqslant . \leqslant \leqq$ by paired t-test.

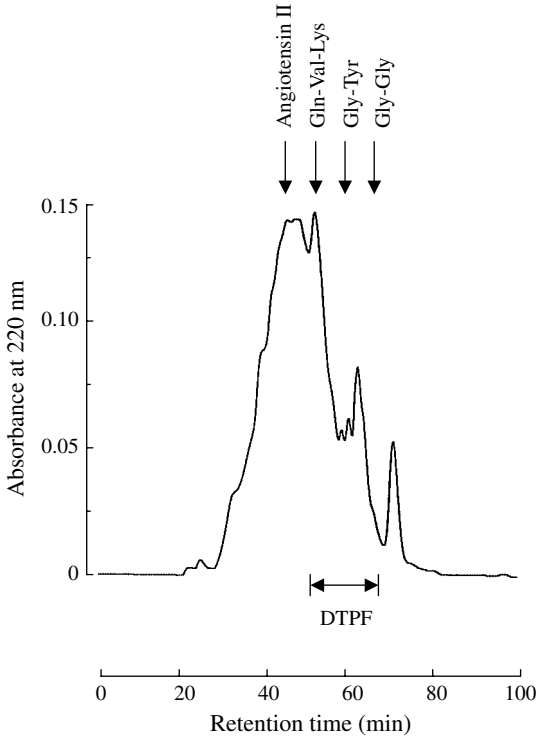

Figure 2 Elution profile by gel permeation mode of HPLC of buckwheat digest. Column: Superdex peptide HR 10/30 (1.0 × 30 cm), elution: 30% CH_3CN containing 0.1% TFA at a flow rate of 0.3 ml/min, detection: 220 nm.

action of intact buckwheat. In contrast, pepsin treatment followed by trypsin and chymotrypsin resulted in a significant increase in the inhibitory activity ($IC_{50:}$ 0.14 mg protein/ml). This indicates that ACE inhibitory peptides were newly produced by the gastrointestinal proteases. The ACE inhibition power of the digest was still low at about 1/9 the level of sardine muscle hydrolysate ($IC_{50:}$ 0.015 mg protein/ml) with an *in vivo* antihypertensive effect. In our previous study, we found that the oral administration of sardine muscle hydrolysate (4 g/day) significantly lowered the blood pressure in mildly hypertensive subjects (Δ systolic BP/Δ diastolic BP after a 4-week protocol: −9.3 mmHg/−5.2 mmHg) [12]. However, considering the amount of buckwheat in a diet, the buckwheat digest would greatly contribute to the reduction in blood pressure. Kawakami *et al.* [16] also demonstrated the positive effect of the enzymatic hydrolysis of buckwheat proteins on the production of ACE inhibitors, where buckwheat globulin after thermolysin hydrolysis had a potent ACE inhibition effect with an IC_{50} of

0.043 mg protein/ml. These findings suggested that intact buckwheat proteins would acquire the ability to inhibit ACE after being consumed and then subjected to gastrointestinal digestion. Rutin and its aglycon, quercetin, did not exhibit any ACE inhibitory activity under our experimental condition of >20 mM, thus agreeing well with the results obtained by Hara *et al.* [17]. Thus, the marked increase in ACE inhibitory activity of buckwheat in protease digestions, as indicated in Table 1, may predominantly be due to those peptides that are newly produced from buckwheat proteins, and not due to rutin.

Antihypertensive Effect of Buckwheat Digest

Figure 1 shows the changes in SBP after the oral administration of buckwheat digest in SHR. Two and four hours after the administration of buckwheat digest, given at a dose of 100 mg/kg, the SBP fell significantly by 27.0±7.6 mmHg ($p < 0.05$) and 29.9±3.4 mmHg ($p < 0.05$). This blood pressure reduction, induced by the ingestion of buckwheat digest, strongly suggested that buckwheat digest has an *in vivo* ability to reduce blood pressure, probably as a result of the peptides that are newly produced from the buckwheat protein. However, further investigations into the dose-dependency of the digest, as well as intact buckwheat or rutin, are needed to clarify the *in vivo* antihypertensive effect.

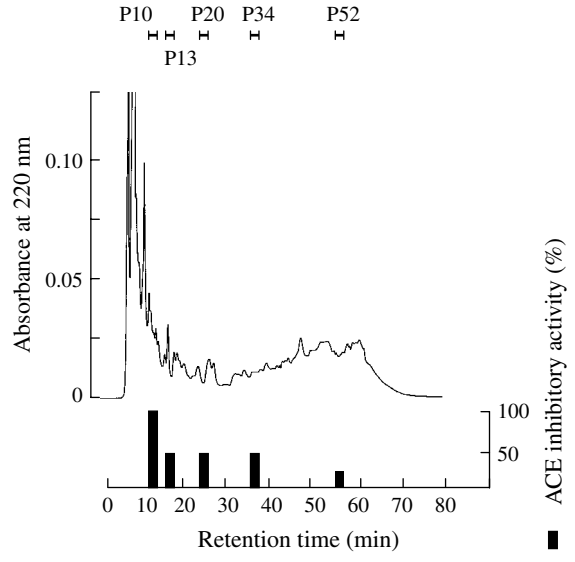

Figure 3 Separation of DTPF eluted from Superdex peptide HR 10/30 column in Cosmosil 5Ph column. Elution was done in a linear gradient mode of CH_3CN (5%–35%, 30 min) containing 0.1% TFA at a flow rate of 0.3 ml/min. Each fraction having ACE inhibitory activity was collected individually.

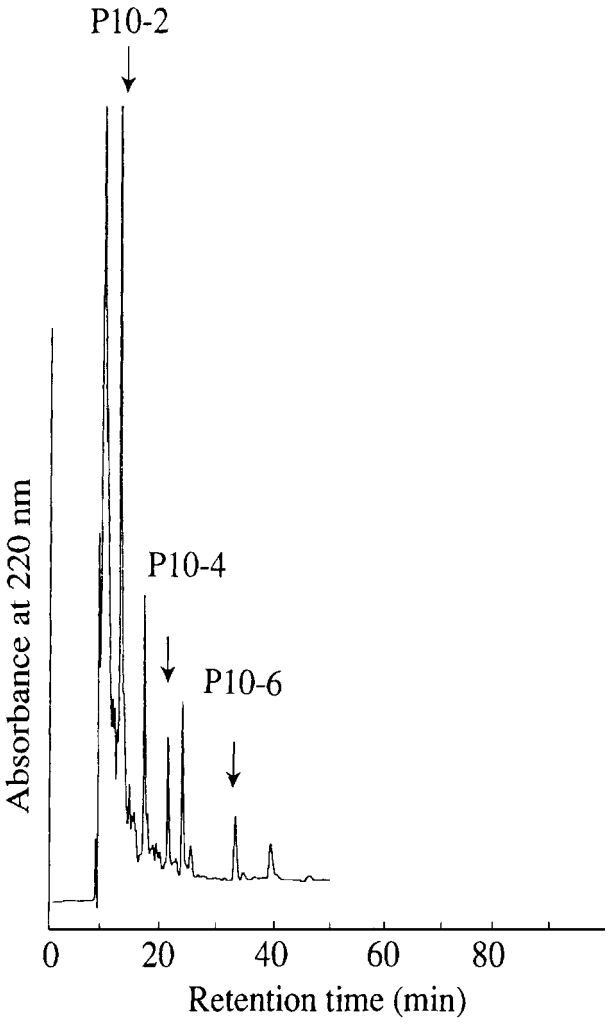

Figure 4 Purification of active fraction P10 in Cosmosil 5C18-ARII column. Elution was done with 5% CH_3CN containing 0.1% TFA at a flow rate of 0.3 ml/min. The P10 fraction was separated into three active peaks denoted as P10-2, P10-4 and P10-6.

Di-/Tri-Peptides in Buckwheat Digest

Based on the evidence of the absorption of di- and tri-peptides through the intestinal mucosal membrane [18], the DTPF in the buckwheat digest was collected to evaluate or identify the ACE inhibitory peptides. As a result of its purification in a Superdex Peptide HR 10/30 column (Figure 2), DTPF was successfully obtained from the buckwheat digest with a yield of 49%. The average peptide length of the DTPF was 2.31, and the IC_{50} value

was 0.22 mg/ml (0.17 mg protein/ml). Then, the magnitude of the ACE inhibitory contribution of the DTPF to the overall inhibition of the buckwheat digest was estimated. After the digestion, 62 mg of DTPF was produced from 1 g of intact buckwheat flour, while the yield of the digest with an IC_{50} value of 0.30 mg/ml was 127 mg/g. Thus, the total ACE inhibitory activity of the DTPF was calculated to be 282 units, while that of the buckwheat digest was 423 units. This indicates that the DTPF derived from the buckwheat digest may be the predominant ACE inhibitory fraction in the buckwheat digest. On the other hand, according to Matsubara *et al.* [19], rutin is a potential antihypertensive substance, given that they observed a strong depressor effect of 48 mmHg after intravenous administration of rutin (1 mg/100 g of body weight) in a stroke-prone SHR. However, the intestinal absorption of rutin into the plasma of Wistar rats was quite slow, even 4 h after administration, so that quercetin was the only detectable plasma metabolite of rutin due to hydrolysis by caecal microflora [20]. Thus, intact absorption or antihypertensive effects of rutin appear unlikely. This also gave rise to speculation that, with a buckwheat diet, the DTPF would mainly retard the action of ACE activity, but not rutin, although the ACE inhibition of quercetin has yet to be studied.

Isolation and Identification of ACE Inhibitors from DTPF

Figure 3 shows the elution pattern of DTPF in a Cosmosil 5Ph column with relative ACE inhibitory activity. Most of the potent ACE inhibitory activity occurred in five fractions, depicted as P10, P13, P20, P34 and P52. Five fractions were then applied to the second step of HPLC in a Cosmosil 5C18-ARII column. Figure 4 shows an example of the HPLC purification of the active fraction of P10. The P10 fraction was separated into three active peaks, namely, P10-2, P10-4 and P10-6. Consequently, 11 active peaks were isolated from the DTPF (Table 2).

The identified peptides, Phe-Tyr [21], Ala-Tyr [9], Leu-Phe [22] and Tyr-Val [23] have already been reported. Koyama *et al.* [24] also found the ACE inhibitory peptide-derivative, Ser-Thr-Hyp in buckwheat with an ACE inhibitory activity of 12.7 μM, exhibiting hypotensive activity in stroke-prone SHR. However, they found the other seven peptides in

Table 2 ACE Inhibitory Peptides Derived from Buckwheat Digest

Amino acid sequence	Amino acid ratio in acid hydrolysate	Peak number[a]	Retention time (min)[a]	IC_{50} (μM)	Yield (%)	Contribution (%)
Val-Lys	Val 1.07, Lys 1.00	P10-2	14.4	13	0.05	3.4
Phe-Tyr[b]	Phe 1.00, Tyr 1.05	P13-9	42.8	25	0.15	3.9
Ala-Tyr[c]	Ala 1.09, Tyr 1.00	P20-2	44.5	100	0.02	0.2
Leu-Phe[d]	Leu 1.63, Phe 1.00	P52-1	81.3	126	0.15	1.0
Tyr-Val[e]	Tyr 1.34, Val 1.00	P13-4	22.2	580	0.15	0.2
Tyr-Gln	Tyr 1.00, Glx 1.33	P10-6	31.4	628	0.15	0.1
Tyr-Gln-Tyr	Tyr 2.13, Glx 1.00	P10-4	20.4	4	0.12	13.6
Pro-Ser-Tyr	Pro 1.31, Ser 1.77, Tyr 1.00	P13-3	16.4	16	0.21	8.1
Leu-Gly-Ile	Leu 1.09, Gly 1.56, Ile 1.00	P34-6	77.3	29	0.08	2.0
Ile-Thr-Phe	Ile 1.19, Thr 1.00, Phe 1.24	P34-9	83.3	49	0.37	4.3
Ile-Asn-Ser-Gln	Ile 1.09, Asx 1.00, Ser 1.11, Glx 1.02	P34-5	75.7	36	0.40	4.0
Total					1.75	40.8

[a] Peak numbers indicate the active peaks eluted from five fractions (P10, P13, P20, P34 and P52) with ACE inhibitory activity. Each peptide was applied in a Cosmosil 5C18-ARII column. Elution was done in a linear gradient mode of CH_3CN (5%–35%, 30 min) containing 0.1% TFA at a flow rate of 0.3 ml/min. Each fraction with ACE inhibitory activity was collected individually.
[b] [21]; [c][9]; [d][22]; [e][23].

natural protein hydrolysate for the first time. The magnitude (%) of the ACE inhibitory contribution of all the identified peptides, relative to the DTPF, was about 41% (Table 2). Therefore, 59% of the active peptides in the DTPF remain unidentified. Tyr-Gln-Tyr and Pro-Ser-Tyr are thought to be the main ACE contributors given the magnitude of their ACE inhibitory contribution.

Cheung *et al.* [9] reported that the hydrophobicity of the carboxyl terminal amino acid was the most important factor affecting the overall binding of the peptides to the active site of ACE. The strong ACE inhibitory activities of Tyr-Gln-Tyr and Pro-Ser-Tyr isolated in this study supported the importance of Tyr at the carboxyl terminal.

CONCLUSIONS

The findings of the current study demonstrate that buckwheat protein has a latent ability to exert an *in vivo* antihypertensive effect after the gastrointestinal digestion process.

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