Angiotensin-Converting Enzyme-Inhibitory Peptides Isolated from Pepsin Hydrolyzate of *Apios americana* Tuber and Their Hypotensive Effects in Spontaneously Hypertensive Rats

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With the aim of elucidating the hypotensive effects of *Apios americana* Medikus tuber, active ingredients with angiotensin-converting enzyme (ACE)-inhibitory activity were identified and their hypotensive effects in spontaneously hypertensive rats (SHR) were investigated. From a pepsin digest of *A. americana* showing strong activity, two ACE-inhibitory peptides were isolated and identified as YRLPNL and YQLP. Although the ACE-inhibitory activity of YQLP was weak, YRLPNL showed strong ACE-inhibitory activity (IC₅₀ value 7.7 nM) equal to those of lisinopril and captopril. After a single oral administration of YRLPNL, SHR showed a significant decrease in blood pressure. These results suggest that YRLPNL, isolated from *A. americana*, is a hypotensive peptide having ACE-inhibitory activity. This is the first report of a food peptide having ACE-inhibitory activity equal to that of pharmaceuticals.

Keywords: *Apios americana*, ACE inhibition, hypotensive effect, peptide, SHR

Introduction

Apios americana Medikus, native to North America, is an edible member of the Leguminosae, and is cultivated in Aomori Prefecture and northern Tohoku region in Japan. Several studies have reported on the nutrients (Walter *et al.*, 1986, Kinugasa and Watanabe, 1992), lipids (Wilson *et al.*, 1986), amino acids (Wilson *et al.*, 1987), DDMP-saponin (Okubo *et al.*, 1994), genistein (Krishnan, 1998), and trypsin inhibitors (Zhang *et al.*, 2008) in the tuber. However, few scientific studies have analyzed the physiological activities of *A. americana* tuber.

A previous study showed a 7% – 11% decline in systolic blood pressure (SBP) in spontaneously hypertensive rats (SHR) fed a diet containing a dried powder of *A. americana* tuber (Iwai and Matsue, 2007). It became clear that *A. americana* showed hypotensive effects in SHR, and we attempted to isolate the active compound(s) from crude hydrophilic and

alcoholic extracts exhibiting some ACE inhibitory activity *in vitro*, as compared to residues without activity. However, we were unable to purify the active substances despite several isolation steps. From these results, we assumed that the ACE inhibitors were derived from *A. americana* during digestion and absorption in the digestive tract.

It is well known that the renin-angiotensin pathway is closely involved in the regulation of blood pressure, and ACE is an important component of this pathway. ACE acts on angiotensin I, which is synthesized from angiotensinogen by renin, and converts it to angiotensin II, which causes the smooth muscle blood vessels to contract, resulting in increased blood pressure. Therefore, ACE inhibitors are useful for managing hypertension.

Ondetti *et al.* (1997) synthesized captopril; a strong ACE inhibitor that is similar to a peptide isolated from snake venom, and successfully used it clinically as an antihypertensive drug. Since Cheung *et al.* (1980) described active peptides showing ACE-inhibitory activity, many studies have been performed to discover ACE-inhibitory peptides in various

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food materials, with the aim of developing functional foods with hypertension-preventive activities. Maruyama *et al.* (1985, 1987) isolated 3 kinds of ACE-inhibitory dodecapeptides from the trypsin hydrolyzate of α -casein and β -casein, and prepared stronger ACE-inhibitory pentapeptides from these dodecapeptides by treatment with other proteases. These studies suggest that the ingestion of ACE-inhibitory peptides is useful in preventing and treating hypertension, and that foods containing ACE-inhibitory peptides will be safe for use by patients with mild hypertension who do not need medical treatment.

Although *A. americana* tuber has been observed to exert an antihypertensive effect in SHR, its active ingredient is not known. Therefore, we aimed to isolate and purify hypotensive ingredients from *A. americana* tuber based on their ACE-inhibitory activity, to identify the molecular structures of the active ingredients and to elucidate the antihypertensive effect of *A. americana*.

Materials and Methods

Reagents Rabbit lung ACE and *N*-hippuryl-histidyl-leucine tetrahydrate (*N*-HHL) were purchased from Sigma Chemical Co. (MO, USA). Porcine pepsin, captopril, and lisinopril were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other reagents were of the highest available grade.

Sample preparation *A. americana* tubers were harvested in November at an Apios farm in Gonohe-machi, Aomori, Japan. They were ground and dried at 60°C.

a) Preparation of *A. americana* pepsin digest (APD)

Ten grams of *A. americana* powder was homogenized in 40 mL of distilled water using a stirrer and extracted at 4°C for 24 h. The mixture was separated into supernatant and residue by centrifugation at 10,000 rpm for 30 min, and the residue was washed with 20 mL of ethanol and dried in a vacuum desiccator. This procedure was repeated 3 times, and all supernatants were collected as the hydrophilic extract and the residue was collected as the *A. americana* residue (AR).

After drying, 5 g of AR was suspended in 50 mL of 0.5 M acetic acid (pH 2.4) and incubated at 37°C for 5 min. Then either 1% or 10% pepsin was added to AR suspensions, which were immediately incubated at 37°C for 24 h. After 24 h, the same amounts of pepsin were added and incubation was repeated. The reaction mixture was centrifuged at 10,000 rpm for 30 min, and the supernatant was collected and boiled for 10 min. The boiled supernatant was centrifuged again and evaporated in a rotary evaporator. Freeze-drying yielded APD.

***b*) Preparation of ACE-inhibitory fraction** APD was fractionated using a Sep-Pak Vac C₁₈ cartridge (20 cc, 5 g;

Waters Co., MA, USA). After the cartridge was conditioned, 200 mg/5 mL of APD solution was applied to the cartridge, followed by stepwise elution with 40 mL of 0%, 20%, 40%, 60%, and 100% acetonitrile (CH₃CN) aqueous solution. These eluates were collected as S1 (0% CH₃CN), S2 (20% CH₃CN), S3 (40% CH₃CN), S4 (60% CH₃CN), and S5 (100% CH₃CN) fractions, respectively, and were evaporated and freeze-dried.

***c*) Isolation and purification of ACE-inhibitory peptides** ACE-inhibitory peptides were isolated from active fractions by high-performance liquid chromatography (HPLC) using a LC-10ADVP pump, SCL-10AVP system controller, CTO-10AV column oven, and SPD-10AVP detector (Shimadzu Co., Kyoto, Japan). CH₃CN and 0.1% trifluoroacetic acid (TFA) solution were eluted through the TSKgel ODS120T [5 μ m; 4.6 mm length (inner diameter) $\times$ 250 mm (length), Tosoh Co., Tokyo, Japan] at room temperature and at a flow rate of 1 mL/min under a linear gradient from 0% to 30% CH₃CN between 0 and 50 min, 30% to 80% CH₃CN between 50 and 51 min, 80% CH₃CN between 51 and 56 min, and 0% CH₃CN between 56.1 and 60 min in 0.1% TFA solution. The eluate was detected at 220 nm and collected in 1-minute fractions.

For purification of peaks using the same HPLC procedure, peptides were eluted at a flow rate of 1 mL/min under a gradient of 40% CH₃CN between 0 and 60 min, 40% to 80% CH₃CN between 60 and 70 min, 80% CH₃CN between 70 and 80 min, and 80% to 40% CH₃CN between 80 and 90 min in 0.1% TFA solution. The eluate was detected at 220 nm and individual peaks were collected.

Synthesis of peptides YQLP, YRLPNL, and their derivatives YRLPN, YRLP, YRL, and YR were synthesized by Fmoc and Boc methods at the Peptide Institute Inc. (Osaka, Japan). Their purities were > 99% by HPLC analysis.

Measurements

***a*) ACE-inhibitory activity** ACE-inhibitory activity was measured by the following procedure based on Cheung *et al.* (1980). Sample and ACE were dissolved in 50 mM borate buffer (pH 8.3), and *N*-HHL as a substrate was dissolved in 50 mM borate buffer (pH 8.3) containing 400 mM NaCl. Briefly, 30 μ L of sample solution was added to 250 μ L of 5 mM *N*-HHL solution and mixed. After incubation at 37°C for 5 min, 100 μ L of 0.01 U/mL ACE solution was added, followed immediately by mixing and incubation at 37°C for 30 min. Then, 250 μ L of 1 N hydrochloric acid was added into the mixture to stop the reaction and 1.5 mL of ethyl acetate was added and vortexed for 1 min. After centrifugation of this mixture at 3,000 rpm for 10 min, 0.5 mL of the ethyl acetate layer was collected into a new tube and the solvent was removed in a centrifugal evaporator. The residue was

dissolved in 4 mL of distilled water and its absorption at 228 nm was measured. The ACE inhibition rate (%) of samples was standardized at 100% of the absorption of enzyme plus substrate without sample. The median inhibitory concentration (IC_{50}) of the sample was calculated from the linear regression of the ACE inhibition rates on the sample concentrations. In the measurements, captopril and lisinopril were used as positive controls.

b) Amino acid content Briefly, 0.3 mL of ninhydrin solution (JEOL Ltd., Akishima, Japan) was added to 0.3 mL of sample solution, followed by mixing and boiling for 15 min. After cooling, 3 mL of 50% methanol solution was added and mixed, and absorption at 570 nm was measured (Friedman, 2004). Amino acid content in the sample was standardized with leucine.

c) Protein content Protein content was measured by the bicinchoninic acid method (Smith *et al.*, 1985) using a BCA Protein Assay kit (Thermo Fisher Scientific Inc., IL, USA). Bovine serum albumin was used as the standard protein.

d) Amino acid and sequence analysis Peptide sample was dissolved in 1 mL of 20% boiling hydrochloric acid and the solution was sealed in a tube under low pressure. After hydrolysis at 110°C for 20 h, the hydrochloric acid was evaporated; the hydrolyzate was then redissolved in 1 mL of distilled water and analyzed with a JLC-500V Automated Amino Acid Analyzer (JEOL Ltd., Akishima, Japan).

After dilution of the sample solution with methanol, the amino acid sequences of peptide samples were determined by Edman degradation using a PPSQ-10 Protein Sequencer (Shimadzu Co., Kyoto, Japan).

e) Mass analysis The masses of peptide samples were measured by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectrometry using a Voyager-DESTR (Applied Biosystems Japan Co., Tokyo, Japan). Angiotensin I (Mw. 1296.6853) and α -cyano-4-hydroxycinnamic acid dimer (Mw. 379.0930) were used as internal standards in the matrix. The measurement parameters in positive reflector mode were: data points, 100,000 channels, 1 nanosecond; scan range, 10 – 2,500; accelerating voltage, 20,000 V; grid voltage, 69.5%; guidewire voltage, 0.002%; delay time, 300 nanoseconds; laser intensity, 1,500;

scans averaged, 256.

f) Nuclear magnetic resonance (NMR) analysis Deuterium oxide (D_2O) permutation, in which peptide samples were dissolved in 99.8% D_2O , was performed twice, after which the samples were dissolved in 99.95% D_2O . After addition of acetone to the sample as the internal standard, 1H -NMR spectra of peptides were measured at 70°C with a JNM EX-270 NMR spectrometer (270 MHz; JEOL Ltd., Akishima, Japan).

Measurement of blood pressure Male SHR was purchased from Japan SLC Inc. (Hamamatsu, Japan). SHR > 12 weeks of age and whose SBP was > 180 mmHg were used. The animal experiments were approved by the Ethics Committee of Aomori University of Health and Welfare, and all rats were treated according to the Guidelines for Animal Experiments of Aomori University of Health and Welfare. The rats were housed individually in stainless wire netting cages in a room maintained at $22 \pm 2^\circ C$, with 65% humidity and 12-hour light/dark cycles.

Captopril as an ACE inhibitor, YRLP, and YRLPNL were dissolved in distilled water at a concentration of 5 mg/mL. All SHR were fasted for 18 h and then divided into control (CNTL), captopril (CAP), YRLP (YRLP), and YRLPNL (YRLPNL) groups of 6 rats each. The CNTL group was given 2 mL/kg of distilled water and the other groups were orally administered 15 mg/kg of captopril, YRLP, and YRLPNL. At 0, 1, 2, 4, 6, and 8 h after administration, the blood pressure of SHR was measured by the tail-cuff method using a NIBP Monitor Model MK-1030 (Muromachi Kikai Co., Tokyo, Japan). Data were reported as mean $\pm$ standard deviation (SD) and compared by the Scheffé test after one-way analysis of variance (ANOVA) using the StatView system (SAS Institute Inc., NC, USA). P values of < 0.05 were considered significant.

Results and Discussion

ACE-inhibitory activity of APD The yield, amino acid and protein contents, and ACE-inhibitory activity of APD prepared by 1% and 10% pepsin hydrolysis are shown in Table 1. Because the higher concentration of pepsin increased APD yield, it was inferred that the soluble hydrolyzate re-

Table 1. Amino acid contents, protein contents, and ACE-inhibitory activities of pepsin digests of *A. americana* tuber residue and hydrophilic extract.

Digest	Yield (%)*	Amino acid content ($\mu g/mg$)	Protein content ($\mu g/mg$)	ACE-inhibitory activity IC_{50} (mg/mL)
1% Pepsin digest	15.18	183.03	409.97	1.3
10% Pepsin digest	28.38	222.70	398.47	1.8
Hydrophilic extract	—	32.92	135.64	30.0

*Yield of digest (APD) was obtained from 5 g of AR.

Table 2. Amino acid contents, protein contents, and ACE-inhibitory activities of fractions from APD by Sep-Pak Vac C₁₈.

Fraction	Yield (%)	Amino acid content (μg/mg)	Protein content (μg/mg)	ACE-inhibitory activity IC ₅₀ (mg/mL)
APD		198.01	342.15	2.00
S1	38.8	147.01	112.27	—
S2	46.4	197.13	518.01	1.50
S3	2.8	116.56	627.97	0.35
S4	Tr	NM	NM	NM
S5	11.8	NM	NM	NM

Tr, trace; NM, not measured; —, could not be calculated.

leased by pepsin from the insoluble protein in AR increased with pepsin level. Protein content decreased and amino acid content increased on addition of pepsin. This suggested that insoluble protein in AR was hydrolyzed by pepsin, increasing soluble peptide fragments and free amino acids, resulting in an increased yield of APD. The ACE-inhibitory activity of APD hydrolyzed with 1% pepsin was stronger than that of APD hydrolyzed with 10% pepsin. This observation suggests that the derived ACE-inhibitory peptides are further eliminated by excess pepsin. APD showed 15 times higher ACE-inhibitory activity than the hydrophilic extract of *A. americana* tuber. This result suggests that pepsin digestion produces novel ACE-inhibitory peptides from insoluble proteins of *A. americana*. ACE inhibition was not observed in hydrolyzates treated with other proteases, such as trypsin and thermolysin (data not shown).

A preliminary experiment using SHR was carried out. Two h after a single oral administration of 200 mg/kg of APD, SBP in SHR decreased by 15 mmHg, after which blood pressure returned to the initial level. In contrast, SBP did not change in SHR that received 200 mg/kg of AR (data not shown). This result suggests that ACE inhibition resulting from pepsin hydrolysis of protein in *A. americana* tuber induced a lowering of blood pressure. Moreover, a moderate antihypertensive effect had been found in SHR fed diets containing *A. americana* tuber powder (Iwai and Matsue, 2007). Therefore, the results suggest that AR had no rapid hypotensive effect, and that pepsin digestion led to the hypotensive effect.

ACE-inhibitory fraction Table 2 shows the yield, amino acid and protein contents, and ACE-inhibitory activity of fractions isolated from APD by Sep-Pak Vac C₁₈. The yield of the S2 fraction, which was eluted with 20% CH₃CN, was highest of all fractions at 46.5%. Amino acid content was highest in S2, and a large amount of protein was isolated from S3, which was eluted with 40% CH₃CN. The S1 fraction, which was eluted with 0% CH₃CN (H₂O), had low levels of protein and was rich in carbohydrates (data not shown). Compared with the ACE-inhibitory activity of APD, that of the S2 and S3 fractions was 1.3 and 5.7 times higher,

respectively. This suggests that ACE-inhibitory peptides are present in both fractions; however, the yield of S3 was small. Since almost all peaks of S3 were detected in the S2 by HPLC analysis, the isolation of ACE-inhibitory peptides was performed using the S2 fraction showing both inhibition and high yield.

Isolation and purification of ACE-inhibitory peptides

Fig. 1 shows the HPLC chromatogram of S2, yields, and ACE inhibition rates of fractions. Many peaks were detected at retention times between 20 and 50 min (Fig. 1-A), but ACE inhibition was observed for peaks S2-29, S2-33, S2-37, S2-41, and S2-50 (Fig. 1-B). Because peak S2-50 showed the strongest ACE inhibition, it was thought to contain ACE-inhibitory peptides, and S2-50 was purified by HPLC.

The HPLC chromatogram and ACE inhibition rates of the purified S2-50 peak are shown in Fig. 2. In the chromatogram produced with UV 220 nm detection, 19 peaks were observed (Fig. 2-A), and ACE inhibition was obtained in peak S2-50-17 at retention time 55 min and S2-50-18 at 60 min (Fig. 2-B). Therefore, these 2 peaks were analyzed structurally with the aim of identifying the ACE-inhibitory peptides of *A. americana* tuber.

Identification of ACE-inhibitory peptides The amino acid sequence of S2-50-18 was determined to be that of YQLP by Edman degradation. S2-50-17 sequence was also determined to be that of YRLP by the same method, but amino acids after the fifth residue could not be determined.

Fig. 1. HPLC chromatogram of peptides in the S2 fraction, showing (A) yields and ACE inhibition rates (B). The HPLC conditions are described in the text. ACE inhibition rate of each fraction was measured at a concentration of 0.2 mg/mL.

Fig. 2. HPLC chromatogram of S2-50 showing purification (A) and ACE inhibition rates (B).

The HPLC conditions are described in the text. ACE inhibition rate of each fraction was measured at a concentration of 10 $\mu\text{g/mL}$.

Therefore, a high-resolution molecular weight determination of S2-50-17 was performed with MALDI-TOF-MS. Fig. 3 shows the MALDI-TOF-MS and MS/MS spectra of S2-50-17 and the MS spectrum of synthesized YRLPNL. In the MS spectrum of S2-50-17, m/z 775.4724 was assigned as $[\text{M}+\text{H}]^+$ of this peptide, and was m/z 227.1331 greater than that of YRLP (Mw. 548.3393). Moreover, in the MALDI-TOF-PSD-MS spectrum (Poepstorff and Fohlman, 1984), S2-50-17 showed a strong signal at m/z 662.44, corresponding

to the elimination of leucine from the molecular ion $[\text{M}+\text{H}]^+$, and a weak signal at m/z 548.75, corresponding to the elimination of asparagine from the strong signal at m/z 662.44. Therefore, S2-50-17 was concluded to be YRLPNL, and the MALDI-TOF-MS spectrum of synthesized YRLPNL was acquired. The high-resolution mass and MS/MS spectra of synthesized YRLPNL were identical to those of S2-50-17. The $^1\text{H-NMR}$ spectrum of S2-50-17 was also identical to that of synthesized YRLPNL and all proton signals were assigned.

From these results, the molecular structure of the ACE-inhibitory peptides S2-50-17 and S2-50-18 originating from *A. americana* tuber hydrolyzate were conclusively identified as YRLPNL and YQLP, respectively.

ACE-inhibitory activity of peptides isolated from *A. americana* With the aim of elucidating the ACE-inhibitory activities of peptides isolated from *A. americana* hydrolyzate, YRLPNL, YQLP, and derivatives of YRLPNL (YRLPN, YRLP, YRL, and YR), derived by elimination of an amino acid residue from the C-terminal end of YRLPNL, were synthesized and assayed. Table 3 shows the ACE-inhibitory activities of these peptides. The activity (IC_{50}) of YQLP as S2-50-18 was $> 5.8 \mu\text{M}$ and was very weak. The derivatives YRLPN, YRL, and YR also showed weak activities of 4.5 μM , 5.6 μM , and 3.9 μM , respectively. However, the activity of YRLP as a derivative of S2-50-17 was 0.29 μM , 20 times that of YQLP. Its activity was similar to that of LRP,

Fig. 3. MALDI-TOF-MS spectrum of S2-50-17 (A), its MS/MS spectrum (B), and MALDI-TOF-MS spectrum of synthesized YRLPNL (C). Measurement conditions are described in the text.

Table 3. ACE-inhibitory activities of peptides isolated from *A. americana* tuber and their derivatives in comparison with known ACE-inhibitory peptides and pharmaceuticals.

Peptide		Molecular weight	ACE-inhibitory activity IC ₅₀		Origin
			(mg/mL)	(μ M)	
YRLPNL	(Tyr-Arg-Leu-Pro-Asn-Leu)	774.911	0.006	0.0077	<i>A. americana</i>
YRLPN	(Tyr-Arg-Leu-Pro-Asn)	661.753	> 3.00	> 4.5	Synthetic
YRLP	(Tyr-Arg-Leu-Pro)	547.650	0.16	0.29	Synthetic
YRL	(Tyr-Arg-Leu)	450.534	2.50	5.60	Synthetic
YR	(Tyr-Arg)	337.376	1.30	3.90	Synthetic
YQLP	(Tyr-Gln-Leu-Pro)	519.593	> 3.00	> 5.80	<i>A. americana</i>
LRP	(Leu-Arg-Pro)	384.50	0.115	0.30	α -Zein
Lisinopril		441.52	0.0048	0.011	Synthetic
Captopril		217.29	0.0046	0.021	Synthetic

which is derived from corn protein α -zein and showed strong ACE-inhibitory activity in food ingredients (Miyoshi *et al.*, 1991). The ACE-inhibitory activity of YRLPNL was 7.7 nM, the strongest among the synthesized peptides, being 37 times that of LRP and equal to that of lisinopril (Bull *et al.*, 1991) and captopril (Ondetti *et al.*, 1977), which have been used clinically as ACE-inhibitory pharmaceuticals. Thus, to the best of our knowledge, this is the first report of a food-derived peptide having ACE-inhibitory activity equal in strength to that of pharmaceuticals.

With the aim of elucidating the relationship between peptide structure and ACE-inhibitory activity, the derivatives resulting from sequential elimination of amino acid residues from YRLPNL were designed and their activities were measured. However, no clear structure-activity relationship was observed. Because enzyme inhibition is dependent on the affinity of the inhibitor to the active site of the enzyme, the elucidation of the inhibitory mechanism of these peptides and their 3-dimensional structural analysis are subjects for future research.

Hypotensive effect of peptides isolated from A. americana
YRLP and YRLPNL showed strong *in vitro* ACE-inhibitory activity, equal to those of peptides previously derived from food protein and of ACE-inhibitory pharmaceuticals. Therefore, changes in blood pressure in SHR orally administered these peptides were investigated to identify the hypotensive effects of these peptides.

Time-course changes of blood pressure in male SHR after a single oral administration of YRLP are shown in Fig. 4. The CNTL group, to which distilled water was administered, showed negligible change in blood pressure. In the captopril group (CAP), in which the rats were given 15 mg/kg of captopril, blood pressure was significantly lower than that of the CNTL group at 1–4 h and showed maximal decrease at 2 h after administration. The YRLP group, in which the rats were given 15 mg/kg of YRLP, showed significantly lower

blood pressure than the CNTL group at 2–4 h after administration. However, the rats in the YRLP group showed a tendency to higher blood pressure than those in the CAP group, with a significant difference in SBP between the YRLP and CAP groups at 2 h after administration. Thus, YRLP lowered blood pressure in SHR, but its hypotensive effect was not as marked as that of ACE-inhibitory pharmaceuticals.

Fig. 5 shows a time course of changes of blood pressure in SHR after a single oral administration of YRLPNL. The blood pressure decrease in the YRLPNL group given 15 mg/kg of YRLPNL was lower than that in the CAP group and showed a maximal decrease at 4 h after administration. The YRLPNL group showed significantly lower SBP and MBP than the CNTL group at 2–6 h after administration, and starting at 6 h, the group showed a tendency for lower blood pressure than the CAP group. This result shows that the hypotensive effect of YRLPNL was somewhat weaker but persisted longer than that of captopril.

Although direct comparison of hypotensive effects between YRLP and YRLPNL is difficult, it is suggested that the hypotensive effect of YRLPNL is stronger than that of YRLP. This is because the differences in blood pressure between the YRLPNL and CNTL groups were larger than those between the YRLP and CNTL groups, and the differences between the YRLPNL and CAP groups were smaller than those between the YRLP and CAP groups.

To date, ACE-inhibitory activities have been observed in hydrolyzates derived from food proteins, and many ACE-inhibitory peptides have been identified. Hypotensive effects of ACE-inhibitory peptides from royal jelly (Tokunaga *et al.*, 2004), egg white hydrolyzate (Miguel *et al.*, 2005), and jellyfish (Morinaga *et al.*, 2010) were observed in SHR following a single oral administration. The 2 peptides isolated from *A. americana* tuber hydrolyzate in the present study showed hypotensive effects in SHR equal to or greater than those isolated previously. LRP obtained after the hydrolysis of

Fig. 4. Time-course changes of blood pressure in SHR after a single oral administration of captopril and YRLP.

SBP, systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure; HR, heart rate. CNTL, control group (3 mL/kg water); CAP, captopril group (15 mg/kg captopril); YRLP, YRLP group (15 mg/kg YRLP). Rats were fasted for 18 h before administration. Data represent the mean $\pm$ SD of 6 rats. Significant differences are indicated from ^aCNTL and ^bCAP groups at a P value of < 0.05.

Fig. 5. Time-course changes of blood pressure in SHR after a single oral administration of captopril and YRLPNL.

SBP, systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure; HR, heart rate. CNTL, control group (3 mL/kg water); CAP, captopril group (15 mg/kg captopril); YRLPNL, YRLPNL group (15 mg/kg YRLPNL). Rats were fasted for 18 h before administration. Data represent the mean $\pm$ SD of 6 rats. ^a Significant differences are indicated from CNTL group at a P value of < 0.05.

the corn protein α -zein (Miyoshi *et al.*, 1991), which shows strong ACE-inhibitory activity, has been reported to lower blood pressure in SHR. However, the blood pressure returned immediately to the initial level following intravenous administration. We suggest that YRLP, whose ACE-inhibitory activity was similar to that of LRP, may be superior to LRP in its hypotensive effect.

A future subject of research is the question of whether the YRLPNL hexapeptide is absorbed intact or is hydrolyzed in the digestive tract. We believe that if YRLPNL is hydrolyzed in the digestive tract, it will exert hypotensive effects, given that YRLP lowers blood pressure in SHR. Our results suggest that it is the ACE-inhibitory activities of YRLPNL and YRLP that affect the blood pressure of SHR fed *A. americana* tuber and that these peptides are beneficial for the prevention of hypertension. The smaller peptide, YRLP, showed lower ACE inhibitory activity than the larger YRLPNL. Nevertheless, the smaller peptide exerted blood pressure lowering activity in SHR, which may imply that another mechanism than ACE inhibition might play a significant role in the

blood pressure lowering effect. Two mechanisms, inhibition of angiotensin II production and inhibition of bradykinin decomposition, are thought to participate in the reduction of blood pressure by ACE inhibitory peptide. ACE is a dicarboxypeptidase that has extended substrate specificity, and it reacts with many endogenous peptides besides angiotensin I as substrates. Accordingly, if peptides capable of acting as a substrate for ACE exist in food-derived protein hydrolyzates, it follows that these hydrolyzates will exhibit ACE inhibitory activity. Moreover, there are some reports that the actual hypotensive effect of peptides, which were isolated from protein hydrolyzates showing ACE inhibitory activity, was induced by other hypotensive mechanisms, such as vasorelaxation, besides ACE inhibitory activity (Yang *et al.*, 2003; Zhao *et al.*, 2008; Yamada *et al.*, 2010). Therefore, the elucidation of other mechanisms regulating the hypotensive effect of YRLP in SHR is a subject of future research.

In this study, it was found that *A. americana* tuber exerted strong ACE-inhibitory activity following 1% pepsin digestion, and YRLPNL and YQLP were identified as strong

ACE-inhibitory peptides in the tuber hydrolyzate. The ACE-inhibitory activity of YRLP was similar to that of LRP derived from the corn protein α -zein, and the activity of YRLPNL was equal to or higher than that of lisinopril and captopril. These peptides also exhibited hypotensive effects in SHR after a single oral administration. This is the first known report of peptides derived from food protein hydrolyzate that showed as strong ACE-inhibitory activity as that of ACE-inhibitory pharmaceuticals. It is further suggested that these peptides play an important role in the hypotensive effect of *A. americana* tuber.

Future research should investigate the absorption of YRLPNL in SHR with the aim of elucidating the mechanism of this hexapeptide in ACE inhibition. The other ACE-inhibitory activities observed in the *A. americana* tuber hydrolyzates suggest the presence of ACE-inhibitory peptides apart from YRLPNL, YQLP, and YRLP. Future research should aim to isolate and identify new ACE-inhibitory peptides and elucidate the characteristics of *A. americana* tuber proteins.

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