JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Purification and Identification of Antihypertensive Peptides from Fermented Buckwheat Sprouts

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ABSTRACT: Buckwheat (*Fagopyrum esculentum*) is rich in antihypertensive compounds. This study investigated the effect of lactic-fermented buckwheat sprouts (neo-FBS) on level, identification, and potency of blood pressure-lowering (BPL) compounds. A single oral dose of 1.0 mg/kg body weight buckwheat sprouts (BS) in spontaneously hypertensive rats did not show significant BPL activity, whereas neo-FBS significantly decreased blood pressure. HPLC of neo-FBS identified two peaks absent in the profile of BS. The peak exhibiting potent BPL activity was fractionated, and six peptides (DVWY, FDART, FQ, VAE, VVG, and WTFR) and tyrosine were identified by LC-MS/MS and Edman degradation. Single oral dose administration of the peptides revealed significant BPL effect of all the peptides, with the most potent being DVWY, FQ, and VVG. DVWY, VAE, and WTFR are novel. This study demonstrates that lactic fermentation of BS produces new, highly potent antihypertensive peptides and increases active compounds GABA and tyrosine already present in BS.

KEYWORDS: *peptide, blood pressure-lowering effects, fermented buckwheat sprouts*

1. INTRODUCTION

Over the past decade, there has been a growing interest in the use of buckwheat for the treatment of hypertension. This pseudocereal is rich in blood pressure-lowering (BPL) compounds[1] such as rutin[2] and γ-aminobutyric acid (GABA).[3] In spontaneously hypertensive rats (SHRs), rutin improves blood vessel elasticity,[4] whereas GABA lowers blood pressure.[5] Buckwheat also contains a very potent angiotensin I-converting enzyme (ACE) inhibitor, 2″-hydroxynicotianamine, with an IC_{50} of 0.08 μM.[6] Accordingly, buckwheat is among the most investigated cereals for the extraction of functional compounds for the treatment of hypertension and related cardiovascular diseases. These BPL compounds are generally extracted from buckwheat sprouts, where they are more abundant than in unsprouted seeds[6−8] because various biochemical reactions triggered by germination could promote their production.[7] In fact, it has been reported that germinated buckwheat grains, which contain 1.92 times more rutin than buckwheat seeds, exert antihypertensive effects in SHRs at a dose of 600 mg/kg/day for 5 weeks.[9]

We developed a new lactic fermentation foodstuff called "fermented buckwheat sprouts (FBS)".[10] FBS had *in vitro* ACE inhibitory activity and exhibited significant systolic blood pressure (SBP) and diastolic blood pressure (DBP) lowering effects in SHRs in a single oral administration test at a dose of 0.10 mg/kg body weight (BW).[11] We further refined this fermentation process to generate neo-FBS, a product that significantly decreased the SBP and DBP in SHRs at a single oral dose of 0.010 mg/kg BW. Dual effects of tissue ACE inhibition and vasorelaxation of neo-FBS were responsible for the BPL action.[12] This potent effect could be caused by higher concentrations of existing BPL compounds or by new BPL compounds formed during the fermentation process. However, the BPL compounds present in neo-FBS have not been identified.

The purpose of this study was to identify the BPL compounds present in neo-FBS and test their BPL activity in SHRs by a single oral administration.

2. MATERIALS AND METHODS

2.1. Chemicals. α-cyano-4-hydroxycinnamic acid (CHCA), diethyl ether, formic acid, GABA, hydrochloric acid (HCl), HPLC grade acetonitrile, HPLC grade methanol (MeOH), phenyl isothiocyanate (PITC), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium hypochlorite (NaOCl), and sodium sulfate (Na_2SO_4) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Dimethylformamide (DMF), piperidine (PPD), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), H-Gln-O*t*Bu·HCl, Boc-Phe-OH, Fmoc-Ala-OH·H_2O, Fmoc-Arg(Pbf)-OH·nIPE, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Glu(O*t*Bu)-OH·H_2O, Fmoc-Gly-OH, Fmoc-Phe-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-OH, and Fmoc-Val-OH were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan), and dichloromethane (DCM), diisopropylethylamine (DIEA),

Received: December 4, 2012
Revised: February 19, 2013
Accepted: February 22, 2013
Published: February 22, 2013

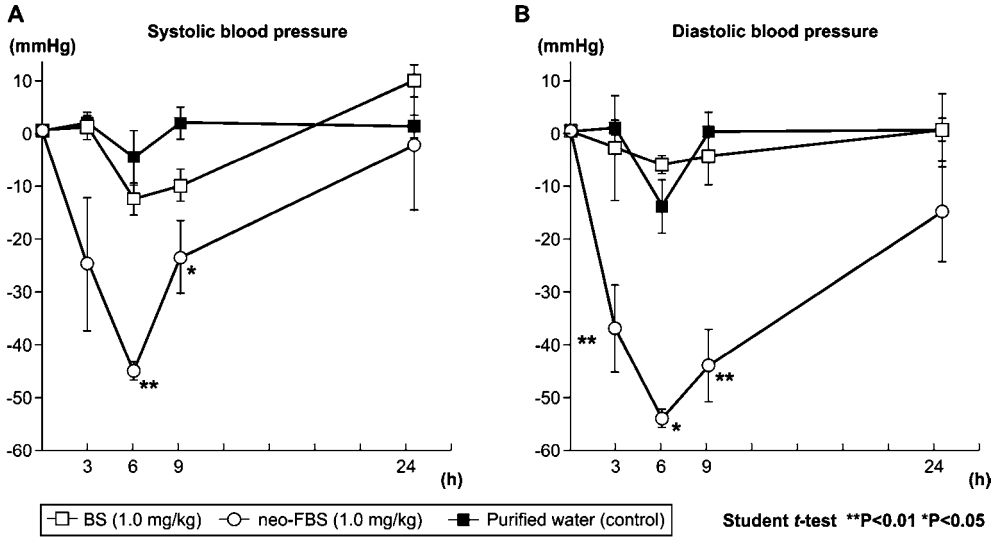

Figure 1. Changes in systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) of 13-week-old spontaneously hypertensive rats (SHRs) after a single oral administration of buckwheat sprouts (BS) and lactic-fermented buckwheat sprouts (neo-FBS) at a dose of 1.0 mg/kg BW. □, BS (n = 6); ○, neo-FBS (n = 6); ■, purified water (control) (n = 6). *p < 0.05; **p < 0.01, versus the control group at each time point as evaluated by Student's t-test.

4-(4,6-dimethoxy-1,3,5-triazine-2-yl)-4-methylmorpholinium chloride (DMTMM), *O*-(benzotriazol-l-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBt), and *N*-methylpyrrolidone (NMP) were purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). Rink Amide AM resin was purchased from Merck Ltd. (Tokyo, Japan).

2.2. Preparation of BS and Neo-FBS Powders. *2.2.1. BS Powder.* Buckwheat sprouts (BS) were provided by Saladcosmo Co. Ltd. (Gifu, Japan) and cultivated. Harvested BS (25.0 g) were sterilized by 100 ppm of NaOCl solution (100 mL) for 10 min and ground with a commercially available juicer. Ground BS were centrifuged at 3.6 × 10^3*g* for 30 min at 4 °C (Centrifuge 5810 R; Eppendorf Co. Ltd., Tokyo, Japan). The precipitate was removed, and the supernatant was lyophilized using a freeze-dryer (EYELA FDU-2000; Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The dry powder (0.27 g) was used for subsequent analyses as lyophilized BS.

2.2.2. Neo-FBS Powder. Neo-FBS powder was prepared as previously reported.[12] Harvested BS (10.0 kg) were sterilized by 100 ppm of NaOCl solution (20.0 L) for 10 min. After rinsing with water, the sterilized BS were cut into 2 cm long pieces and ground with the juicer. A starter solution of *Lactobacillus plantarum* KT (Biotech Japan, Agano, Japan) was added to the ground BS at a rate of 25.0 mL/kg of BS and stored in a tightly covered container (15.0 L). After expelling the air in the container, the fermentation process was started under an inert nitrogen (N_2) gas atmosphere. After 2 weeks of fermentation at room temperature, 8.10 kg of neo-FBS product was obtained. The product was squeezed, and the resultant liquid was centrifuged at 3.6 × 10^3*g* for 30 min at 4 °C (centrifuge 5810 R) to obtain a pink-colored supernatant (6.80 kg). The liquid neo-FBS product was lyophilized using a freeze-dryer. The red-colored powder (137.4 g) was used for subsequent analyses as lyophilized neo-FBS.

2.3. HPLC Analysis. BPL compounds were suspected to be more concentrated in neo-FBS, and HPLC analysis was used to investigate. To pick up the candidate peaks, HPLC analyses of neo-FBS and BS were performed through an ODS column that has been successfully applied to food analysis. One milligram each of lyophilized BS or neo-FBS was dissolved in 1.0 mL of 0.1% TFA in water. Separation was performed at 30 °C using a Chemcobond 5-ODS-W reversed phase column (4.6 × 150 mm; ChemcoPlus Scientific Co., Ltd., Kyoto, Japan) with isocratic elution. Elution was performed at a flow rate of 0.8 mL/min using the 0.1% TFA water. Spectrophotometric detection was performed at 215 nm, and the injection volume was 20 μL. ODS-peaks 1 and 2 were detected as the candidate peaks (Figure 2).

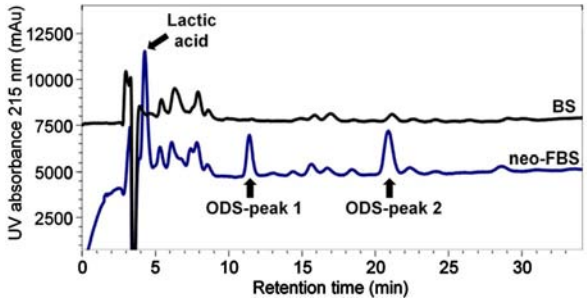

Figure 2. HPLC chromatograms at UV 215 nm of buckwheat sprouts (BS) and lactic-fermented buckwheat sprouts (neo-FBS). Separation was performed using a 4.6 × 150 mm ODS column (Chemcobond 5-ODS-W). The two new peaks on the neo-FBS trace increased by the fermentation process, namely, ODS-peak 1 (11.5 min) and ODS-peak 2 (21.0 min). The peak at 4.5 min was lactic acid.

2.4. Separation and Purification of BPL Compounds in Neo-FBS. Initially, active fractions in neo-FBS were separated and collected by solid phase extraction. Briefly, 1.0 mL of neo-FBS samples in water (50 mg/mL) was eluted on a Sep-Pak Vac C_{18} 10 g cartridge (Waters Co., Milford, MA, USA) in 100 mL of methanol/0.1% TFA water (10:90 v/v) at room temperature. Every 1.0 mL fraction from #1 to #100 was collected and every aliquot was analyzed with HPLC to confirm the presence of the candidate peaks. The combined fractions #37 to #78 were evaporated under vacuum and lyophilized for further analysis.

The lyophilized samples were dissolved in the 0.1% TFA water to a concentration of 50 mg/mL and then purified by HPLC through a Chemcobond 5-ODS-W preparative column (20 × 250 mm; ChemcoPlus Scientific Co., Ltd.) with isocratic elution to fractionate ODS-peaks 1 and 2. Elution was performed with the 0.1% TFA water at 30 °C with a flow rate of 10 mL/min. Absorbance was detected at 215 nm, and the injection volume was 500 μL. Fractionated ODS-peaks 1 and 2 were evaporated under vacuum and lyophilized and were then used in animal experiments.

To further purify compounds in the lyophilized ODS-peak 2, we investigated HPLC conditions using various separation mode columns such as ODS columns with higher or lower carbon content than those of the ODS column used in first chromatographic dimension, a gel filtration column, an amino column, an amide column, and a

hydrophilic interaction column. The best separation of compounds in ODS-peak 2 was achieved using the amide column. Separation using the amide column was performed at 30 °C using a 4.6 × 250 mm amide column (TSKgel Amide-80; Tosoh Co., Tokyo, Japan). Gradient elution was performed at a flow rate of 0.8 mL/min using two mobile phases of the 0.1% TFA water (solvent A) and acetonitrile (solvent B): 0−15 min, isocratic 5% solvent A; 15−20 min, gradient 5−10% solvent A; and 20−80 min, gradient 10−25% solvent A. Chromatography was performed with an injection volume of 20 μL, and UV detection was done at 215 nm. These HPLC analytic conditions were used in LC-MS/MS analysis of fractionated ODS-peak 2.

2.5. Identification of Peptides in Neo-FBS Using LC-MS/MS Analysis and Amino Acid Sequencing Analysis. *2.5.1. LC-MS/MS Analysis.* LC-MS/MS analysis was performed at the CREFAS (Collaborated Research Center for Food Functions, Faculty of Agriculture, Shinshu University). Compounds in ODS-peak 2 were analyzed using a LC-MS/MS system of a Quattro micro API (MS) system with a Waters 2695 (LC) (Waters Co.). The HPLC conditions were the same as described for analysis of ODS-peak 2 using the amide column. Mass spectra were acquired in electrospray ionization (ESI) mode using 3500 V of capillary voltage, 20 V of cone voltage, desolvation gas (N_2) flow of 350 L/h, cone gas (N_2) flow of 50 L/h, source temperature of 100 °C, and desolvation temperature of 350 °C. The mass spectrometer was operated in positive mode and with a scanning range of m/z 100−1000. The precursor ions (m/z 274.2, 294.2, 318.0, 582.5, 609.5, and 609.6) selected by MS1 were fragmented by collision-induced dissociation using argon gas at a flow rate of 9.0 mL/h and collision voltage of 30 V. In lyophilized BS, the presence of six identified ion peaks was analyzed by single-ion monitoring mode in the LC-MS conditions described above.

2.5.2. Amino Acid Sequencing Analysis. The six fractions collected from HPLC fractionation of ODS-peak 2 in the amide column were dried and analyzed by amino acid sequencing. Amino acid sequence analysis was performed at Nippi Inc. (Tokyo, Japan) by stepwise Edman degradation using an automated gas phase sequencer (Procise492HT; Applied Biosystems, Inc., CA, USA) coupled with HPLC identification of the resulting phenylthiohydantin-amino acids. Amino acid sequences of the peptides contained in each fraction were determined, and the results supported those of the LC-MS/MS analyses.

2.6. Quantification of GABA and Tyrosine in BS and Neo-FBS. *2.6.1. GABA.* The GABA present in BS, neo-FBS, and a standard solution was derivatized to phenylthiocarbamyl-GABA (PTC-GABA) as previously described.[13] Briefly, lyophilized BS and neo-FBS were diluted to 100 mg/mL, whereas commercially available GABA was diluted to 125 mM, all in purified water. Then a 20 μL aliquot of supernatant (or of standard solution) was dried under vacuum. The residue was dissolved in 10 μL of ethanol/water/triethylamine (2:2:1 v/v/v) and dried under vacuum. A 20 μL volume of ethanol/water/triethylamine/PITC (7:1:1:1 v/v/v/v) was added to the residue and allowed to react for 20 min at room temperature to form PTC-GABA. Excess reagent was then removed under vacuum. The dry residue containing PTC-GABA was dissolved in the mobile phase and was analyzed by HPLC.[14] Separation was performed at 40 °C using a Mightysil RP-18 GP aqua reversed phase column (4.6 × 250 mm; Kanto Chemical Co., Inc. Tokyo, Japan). Gradient elution was performed at a flow rate of 0.8 mL/min using two mobile phases of acetonitrile/acetic acid buffer (pH 6.5) (25:975 v/v, solvent A) and acetonitrile/water/methanol (450/400/150 v/v/v, solvent B); 0−50 min, gradient 5−40% solvent B. Detection was performed using UV spectrophotometry at 254 nm using an injection volume of 10 μL. The amount of GABA in samples was calculated using a calibration curve. The linear calibration curve for GABA was obtained in the concentration range 0.05−0.5 mM with a correlation coefficient of 0.9999. Linearity was described by the equation $y = 9 \times 10^8 x - 5582.8$. All measurements were performed in triplicates, and the results were expressed as mean ± standard error (SE).

2.6.2. Tyrosine. One milligram each of lyophilized BS and neo-FBS was dissolved in 1.0 mL of the 0.1% TFA water. Separation was performed at 30 °C using a 4.6 × 250 mm amide column (TSKgel Amide-80). Gradient elution was performed at a flow rate of 0.8 mL/min using two mobile phases of the 0.1% TFA water (solvent A) and acetonitrile (solvent B): 0−15 min, isocratic 5% solvent A; 15−25 min, gradient 5−10% solvent A. UV detection was performed at 215 nm with an injection volume of 20 μL. The amount of tyrosine in samples was calculated using a calibration curve. The linear calibration curve for tyrosine was obtained in the concentration range 4.31−17.3 μM with a correlation coefficient of 0.9962. Linearity was described by the equation $y = 3.47 \times 10^4 x - 8001.2$. All measurements were performed in triplicates, and the results were expressed as mean ± SE.

2.7. Synthesis of Peptides Isolated from Neo-FBS. The six peptides identified by LC-MS/MS analyses and Edman degradation were synthesized to test their BPL effect *in vivo*. FQ was synthesized by a stepwise liquid phase peptide synthesis method, and VAE, VVG, DVWY, WTFR, and FDART were synthesized by a 9-fluorenylmethyloxycarbonyl (Fmoc)-solid phase peptide synthesis method in our laboratory. To prepare FQ, Boc-Phe-OH (3.0 mmol, 0.79 g) dissolved in MeOH (3.0 mL) was added to DMTMM (1.5 equiv, 1.51 g) and stirred for 10 min. The reaction mixture was then added to H-Gln-O*t*Bu (3.0 mmol, 0.72 g) and stirred for 1 h. Ethyl acetate (50 mL) and purified water (30 mL) were added to the reaction mixture. The aqueous layer was removed, and the organic layer was washed with 1 N HCl (30 mL), 1 N NaOH (30 mL), and saturated saline. After drying over anhydrous Na_2SO_4, the organic layer was evaporated to dryness under reduced pressure. The dry residue was purified by flash column chromatography (DCM/MeOH = 9:1 as eluent) to give Boc-Phe-Gln-O*t*Bu (84% yield). This product (2.5 mmol, 1.12 g) was dissolved in DCM (3.0 mL) containing TIPS (60 μL). The mixture was added to TFA (1.5 mL) and stirred for 10 h. Diethyl ether (50 mL) was added, and then the reaction mixture was filtered to obtain a precipitate. The precipitate was rinsed with diethyl ether (30 mL), and after drying under reduced pressure, crude H-Phe-Gln-OH (FQ) was obtained (70% yield).

VAE was synthesized using a personal multiple synthesizer (PetiSyzer; HiPep Laboratories, Kyoto, Japan). Fmoc-Glu(O*t*Bu)-OH (1.2 mmol, 0.53 g) was mixed with a condensing agent (HBTU, 1.2 mmol, 0.46 g; HOBt, 1.2 mmol, 0.16 g; DMF, 2.7 mL), and then 0.9 M DIEA (DIEA, 0.42 mL; NMP, 2.3 mL) was added before being stirred for 10 min. The reaction mixture was added to solid phase resin (Rink Amide AM resin; Merck Ltd., Tokyo, Japan, 500 mg), stirred for 8 h at 50 °C, and then cooled at room temperature. The resin was washed with DCM/DMF (1:1, 50 mL) to obtain Fmoc-Glu(O*t*Bu)-resin. The resin was added to Fmoc deprotection agent (PPD, 12.0 mM; DMF, 5.4 mL) and stirred for 20 min at room temperature. The deprotected resin was washed with DMF (25 mL) to obtain H-Glu(O*t*Bu)-resin. Following a similar procedure to that above, H-Val-Ala-Glu(O*t*Bu)-resin was synthesized using Fmoc-Ala-OH (1.2 mmol, 0.40 g) and Fmoc-Val-OH (1.2 mmol, 0.41 g). The resin was added to deprotection−deresination agent (TFA/H_2O/TIPS, 95:2.5:2.5, 10 mL) and stirred for 90 min. After removing the resin by filtration, diethyl ether (40 mL) was added to the filtrate, and then the reaction mixture was centrifuged at $3.6 \times 10^3 g$ for 30 min at 4 °C (centrifuge 5810 R) to obtain a precipitate. The precipitate was rinsed with diethyl ether (30 mL), and after drying under reduced pressure crude H-Val-Ala-Glu-OH (VAE) was obtained (66% yield). VVG, DVWY, WTFR, and FDART were synthesized using a method similar to that used for VAE synthesis. VVG was synthesized using Fmoc-Val-OH and Fmoc-Gly-OH, and the crude peptide was obtained in 92% yield. DVWY was synthesized using Fmoc-Asp(O*t*Bu)-OH, Fmoc-Val-OH, Fmoc-Trp-(Boc)-OH, and Fmoc-Tyr(O*t*Bu)-OH, and the crude peptide was obtained in 36% yield. WTFR was synthesized using Fmoc-Trp(Boc)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Phe-OH, and Fmoc-Arg(Pbf)-OH, and the crude peptide was obtained in 57% yield. FDART was synthesized using Fmoc-Phe-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, and Fmoc-Thr(*t*Bu)-OH, and the crude peptide was obtained in 47% yield.

The crude peptides (FQ, VAE, VVG, DVWY, WTFR, and FDART) were purified by normal phase HPLC through a 21.5 × 300 mm amide column (TSKgel Amide-80). Gradient elution was performed at a flow

rate of 10 mL/min using mobile phases of the 0.1% TFA water (solvent A) and acetonitrile (solvent B): 0−15 min, isocratic 5% solvent A; 15−20 min, gradient 5−10% solvent A; and 20−40 min, gradient 10−15% solvent A. UV detection was performed at 215 nm, and the injection volume was 100 μL. FQ: yield 63%, HPLC purity 96%, MALDI-TOF/MS ($[M + H]^+$) observed mass 294.1465, calculated mass 294.1453. VAE: yield 50%, HPLC purity 95%, MALDI-TOF/MS ($[M + H]^+$) observed mass 318.1697, calculated mass 318.1665. VVG: yield 80%, HPLC purity 96%, MALDI-TOF/MS ($[M + H]^+$) observed mass 274.1756, calculated mass 274.1766. DVWY: yield 14%, HPLC purity 95%, MALDI-TOF/MS ($[M + H]^+$) observed mass 582.2561, calculated mass 582.2563. WTFR: yield 38%, HPLC purity 98%, MALDI-TOF/MS ($[M + H]^+$) observed mass 609.3150, calculated mass 609.3149. FDART: yield 26%, HPLC purity 97%, MALDI-TOF/MS ($[M + H]^+$) observed mass 609.2990, calculated mass 609.2996. MALDI-TOF/MS (AB SCIEX TOF/TOF 5800; AB Sciex, CA, USA) was used to confirm the molecular masses of the synthesized peptides. The peptide mass spectra were acquired in reflector positive ion mode with an accelerating voltage of 20 kV using CHCA as matrix. Laser shots of 750 per spectrum were used to obtain the spectra. On the basis of the signal-to-noise (S/N) ratio, the spectrum of each peptide was obtained from the cumulative spectra after laser bombardment of 750 times.

2.8. Examination of Antihypertensive Effects in SHRs. Animal studies were performed using 13-week-old male SHRs weighing between 300 and 350 g as previously described.[12] The SHRs (Charles River Laboratories Japan, Inc., Kanagawa, Japan) were allowed one week acclimatization in independent cages. They were given free access to commercial laboratory feed (MF; Charles River Laboratories Japan, Inc.) and tap water in a controlled temperature room (22−23 °C) with a 12 h light−dark cycle (lights on 8:00−20:00). Two groups of SHRs received a single oral dose of lyophilized BS or neo-FBS dissolved in purified water (1.0 mg/kg BW). A third control group received only purified water. The lyophilized aliquots of ODS-peaks 1 and 2 were dissolved in purified water and were each administered to six SHRs at a single oral dose of 0.010 mg/kg BW, which was the minimum effective dose of neo-FBS. This was done to investigate whether the fractionated peaks contained adequate BPL compounds in neo-FBS. DVWY, FDART, FQ, VAE, VVG, and WTFR were dissolved in purified water and were administered in a single oral dose of 0.10 mg/kg BW to each of five SHRs to investigate the BPL effect of identified peptides. A dose higher than the minimum effective dose of neo-FBS was employed to investigate the BPL effects. The control group received only purified water. Blood pressure change by administration of the samples was indirectly recorded using the tail-cuff method (Softron BP-98A; Softron Co. Ltd., Tokyo, Japan) at 0, 3, 6, 9, and 24 h after administration. Experimental procedures were performed with the approval of the Animal Care Committee of the Faculty of Agriculture of Shinshu University.

2.9. Statistical Analysis. All results are expressed as mean ± SE. Student's *t*-test was used to determine the significance of differences between groups ($*p < 0.05$, $**p < 0.01$).

3. RESULTS

3.1. Evidence for BPL Effect in BS and Neo-FBS. Neo-FBS significantly decreased SBP (6 to 9 h after treatment) and DBP (3 to 9 h after treatment). However, BS did not show significant SBP- and DBP-lowering effect (Figure 1A and B). Neo-FBS treatment produced a 40 mmHg reduction in both SBP and DBP at 6 h after administration. Since no BPL effect was observed after administration of BS at a 100-fold the minimum effective dose of neo-FBS, this suggests that BS did not contain enough BPL compounds to cause the BPL action, quantitatively and/or qualitatively. Collectively, these data demonstrate that the level of compounds responsible for the BPL action of neo-FBS increased during the lactic fermentation of BS.

3.2. Amount of Known BPL Compounds, GABA and Tyrosine, in BS and Neo-FBS. Known BPL compounds GABA[3] and tyrosine[15] were present in both BS and neo-FBS. Table 1 shows the quantities of GABA and tyrosine in BS and neo-FBS. The quantity of GABA in BS and neo-FBS was determined to be 1.4 ± 0.2 mg/g dry weight (DW) and 2.6 ± 0.2 mg/g DW, respectively. GABA content in neo-FBS was 1.86 times higher ($p < 0.05$) than in BS. The quantity of tyrosine in BS and neo-FBS was 0.54 ± 0.02 and 2.6 ± 0.1 mg/g DW, respectively. Tyrosine content in neo-FBS was 4.89 times higher than in BS. These results indicated that lactic fermentation increases the amount of GABA and tyrosine, which could contribute to the BPL effect of neo-FBS.

Table 1. GABA and Tyrosine Content (mg/g DW) in Buckwheat Sprouts (BS) and Lactic-Fermented Buckwheat Sprouts (neo-FBS)

	GABA	tyrosine
BS	1.4 ± 0.2	0.54 ± 0.02
neo-FBS	2.6 ± 0.2[a]	2.6 ± 0.1[a]

[a] $p < 0.01$, versus the BS as evaluated by Student's *t*-test.

3.3. Identification of New BPL Compounds in Neo-FBS. In order to identify newly formed BPL compounds by lactic fermentation of BS, we initially compared HPLC chromatograms of BS and neo-FBS on an ODS column. This analysis revealed peaks that were peculiar to neo-FBS at 4.5, 11.5, and 21.0 min (Figure 2). The peak at 4.5 min was found to be lactic acid produced by the lactic fermentation. The fractions at 11.5 min (ODS-peak 1) and 21.0 min (ODS-peak 2) were purified by preparative HPLC after solid phase extraction from neo-FBS and lyophilized. Each of the lyophilized fractions was dissolved in purified water and then administered to SHRs in a single oral dose of 0.010 mg/kg BW. Time-course analysis revealed that the fractions from both ODS-peaks caused significant decreases in blood pressure in SHRs, with similar profiles for SBP and DBP (Figure 3A and B). The maximum response to ODS-peak 1 was observed at 6 h, whereas the maximum response to ODS-peak 2 was reached at 9 h postadministration. The fractions from both peaks caused significant reductions in SBP of 30−35 mmHg, compared to the untreated SHRs. In contrast, only ODS-peak 2 significantly lowered DBP by 50 mmHg compared to untreated animals. The difference in BPL activities suggests that these two peaks contain different BPL compounds. Therefore, we purified and identified the superior BPL compounds contained in an aliquot of ODS-peak 2.

3.4. Isolation and Identification of BPL Compounds in Neo-FBS. Figure 4 shows the HPLC chromatogram of ODS-peak 2 with the amide column and indicates six main peaks. Contents of compounds contained in peaks 4 and 6 were relatively abundant in the ODS-peak 2, as judged from the area size of the separated peaks. LC-MS/MS analysis of the separated peaks identified one amino acid (tyrosine) and six oligopeptides with molecular weight of m/z 200−700 (DVWY, FDART, FQ, VAE, VVG, and WTFR) from peaks 1−6. Figure 5 shows the LC-MS/MS spectra and Table 2 shows fragment ion data of identified peptides. All ions observed in the positive ion mode spectra were singly charged $[M + H]^+$ ions, and a-, b-, y-, and z-series fragmentation ions of peptides were well observed as described previously.[16] Determination of amino acid sequences of the peptides present in each aliquot of the

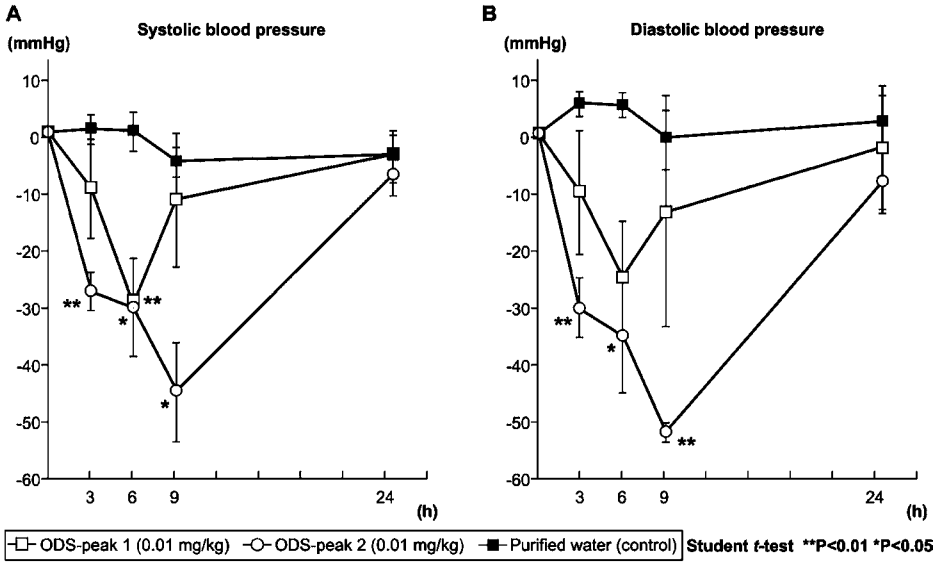

Figure 3. Impact of the fractionated ODS-peaks 1 and 2 of lactic acid-fermented buckwheat sprouts (neo-FBS) on systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) of spontaneously hypertensive rats (SHRs). The fractionated ODS-peaks 1 and 2 were administrated at a single oral dose of 0.010 mg/kg BW. □, ODS-peak 1 (n = 6); ○, ODS-peak 2 (n = 6); ■, purified water (control) (n = 6). *p < 0.05; **p < 0.01, versus the control group at each time point as evaluated by Student's t-test.

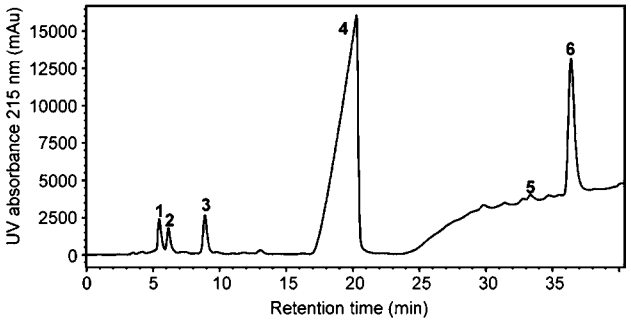

Figure 4. HPLC chromatogram on an amide column of fractionated ODS-peak 2. The column used was a TSKgel Amide-80 (4.6 × 250 mm). Peaks: 1, WTFR; 2, FDART; 3, DVWY; 4, tyrosine; 5, VAE; 6, FQ and VVG.

HPLC peaks was performed by automated Edman degradation, and the results supported those of the MS/MS analyses.

3.5. BPL Effects of Identified Peptides Administered to SHRs at Single Oral Doses. The BPL capacity of the six compounds identified in neo-FBS (DVWY, FDART, FQ, VAE, VVG, and WTFR) was tested in SHRs. They were synthesized and administered to the rats as a single oral dose of 0.10 mg/kg BW. The BPL values of the peptides were calculated with respect to the negative control (purified water) animals. Figures 6 and 7 compare the time-course of SBP and DBP in the control and treated animals. All six compounds exhibited significant BPL activities. DVWY significantly decreased SBP by 54.9 ± 1.3 mmHg (at 3 to 6 h postadministration) and DBP by 27.8 ± 4.3 mmHg (at 9 h postadministration). FDART (26.4 ± 3.7 mmHg at 9 h postadministration) and VAE (17.7 ± 2.0 mmHg at 3 h postadministration) caused significant reductions in DBP only. FQ significantly decreased SBP at 9 to 24 h and DBP at 3 and 9 h postadministration. Maximum BPL of FQ was 33.6 ± 3.4 mmHg in SBP and 41.4 ± 3.5 mmHg in DBP at 9 h postadministration. VVG significantly decreased SBP at 6 to 9 h and DBP at 3 to 24 h postadministration. The BPL effect of VVG was maintained for a prolonged time period. This peptide had SBP- and DBP-lowering values of 28.3 ± 1.5 and 37.1 ± 3.9 mmHg, respectively, at 9 h postadministration. WTFR significantly decreased SBP only by 29.5 ± 4.1 mmHg at 3 to 6 h postadministration. After administration, all blood pressure parameters returned to the levels of untreated animals within 24 h except for FQ and VVG. Collectively, these data demonstrate that we identified six new BPL compounds from neo-FBS, the most potent being DVWY, FQ, and VVG. The ion peaks of identified peptides were not detected in LC-MS analysis of BS, suggesting that the peptides were specifically formed in the fermentation process that produced neo-FBS.

4. DISCUSSION

The medicinal value of buckwheat for the treatment of hypertension depends on the development of efficient extraction methods to isolate and concentrate the BPL compounds. These methods should be able to isolate the active peptides in lactic-fermented buckwheat protein. In the present study, we demonstrate that the fermentation protocol developed for BS dramatically increases the concentration of BPL compounds in BS and isolated new potent BPL peptides from BS protein, which have therapeutic effects in animal studies.

The BPL effects of buckwheat extracts in SHRs have been reported in several studies over the past decade. Our research team focused on developing new extraction methods that would increase the BPL potency of BS by lactic fermentation. In the present study, we show that simple extracts of BS (1.0 mg/kg BW) did not reduce the blood pressure of SHRs. In contrast, extracts from lactic-fermented BS (neo-FBS; 1.0 mg/kg BW) significantly reduced SBP and DBP by 39.9 ± 1.1 and 40.8 ± 1.5 mmHg, respectively. This potency is higher than previously reported for any form of BS extracts in SHRs. For instance, one study[17] showed that enzyme digest of buckwheat decreased SBP by 29.9 ± 3.4 mmHg at a single oral dose of 100 mg/kg, while another[9] study reported that germinated buckwheat grains decreased SBP by 53 mmHg at a dose of 600 mg/kg/day for 5 weeks. These findings suggest that our

dx.doi.org/10.1021/jf305157y | J. Agric. Food Chem. 2013, 61, 3013−3021

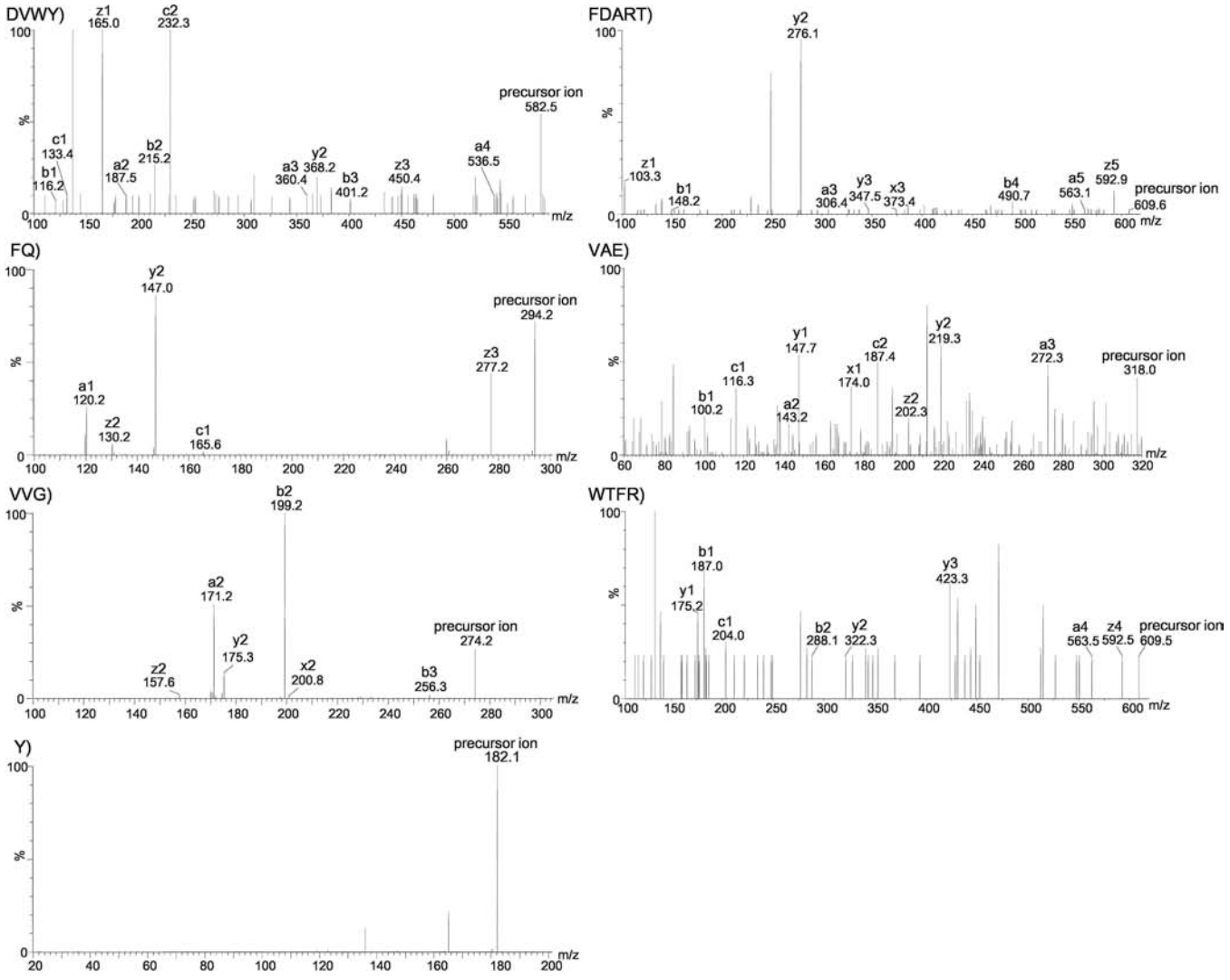

Figure 5. MS/MS spectra of DVWY, FDART, FQ, VAE, VVG, WTFR, and tyrosine. Some a-, b-, c-, x-, y-, and, z-ions derived from the peptides ion are indicated.

Table 2. Fragment Ion Data of Identified Peptides by ESI LC-MS/MS

peak no.	amino acid sequence	observed mass $[M + H]^+$ (m/z)	theoretical mass $[M + H]^+$ (m/z)	fragmentation ions in MS/MS (m/z)
1	H-Trp-Thr-Phe-Arg-OH (WTFR)	609.5	609.3	592.5 (z4), 563.5 (a4), 423.3 (y3), 322.3 (y2), 288.1 (b2), 204.0 (c1), 187.0 (b1), 175.2 (y1)
2	H-Phe-Asp-Ala-Arg-Thr-OH (FDART)	609.6	609.3	592.9 (z5), 563.1 (a5), 490.7 (b4), 373.4 (x3), 347.5 (y3), 306.4 (a3), 276.1 (y2), 148.2 (b1), 103.3 (z1)
3	H-Asp-Val-Trp-Tyr-OH (DVWY)	582.5	582.3	536.5 (a4), 450.4 (z3), 401.2 (b3), 368.2 (y2), 360.4 (a3), 232.3 (c2), 215.2 (b2), 187.5 (a2), 165.0 (z1), 133.4 (c1), 116.2 (b1)
4	Y	182.1	182.1	
5	H-Val-Ala-Glu-OH (VAE)	318.0	318.2	272.3 (a3), 219.3 (y2), 202.3 (z2), 187.4 (c2), 174.0 (x1), 147.7 (y1), 143.2 (a2), 116.3 (c1), 100.2 (b1)
6	H-Phe-Gln-OH (FQ)	294.2	294.1	277.2 (z3), 165.6 (c1), 147.0 (y2), 130.2 (z2), 120.2 (a1)
6	H-Val-Val-Gly-OH (VVG)	274.2	274.2	256.3 (b3), 200.8 (x2), 199.2 (b2), 175.3 (y2), 171.2 (a2), 157.6 (z2)

extraction method using lactic fermentation either increased the concentration of the BPL compounds or induced the generation of new ones.

Quantitative analysis of tyrosine and GABA, which are known BPL compounds in buckwheat, in BS and neo-FBS showed that the levels of both were significantly higher in neo-FBS. It has been reported that free amino acids and GABA were produced by lactic fermentation from food materials.[18,19] BPL effects of tyrosine and GABA are well-established. Indeed, tyrosine significantly reduced SBP by 40 mmHg after intraperitoneal administration at a dose of 200 mg/kg in SHRs.[15] GABA has been reported to have significantly decreased SBP by 15.0 mmHg at a dose of 0.5 mg/kg in SHRs.[5] Neo-FBS, which had significantly higher levels of tyrosine and GABA, had a more potent BPL action than BS in this study. These findings demonstrate that these compounds

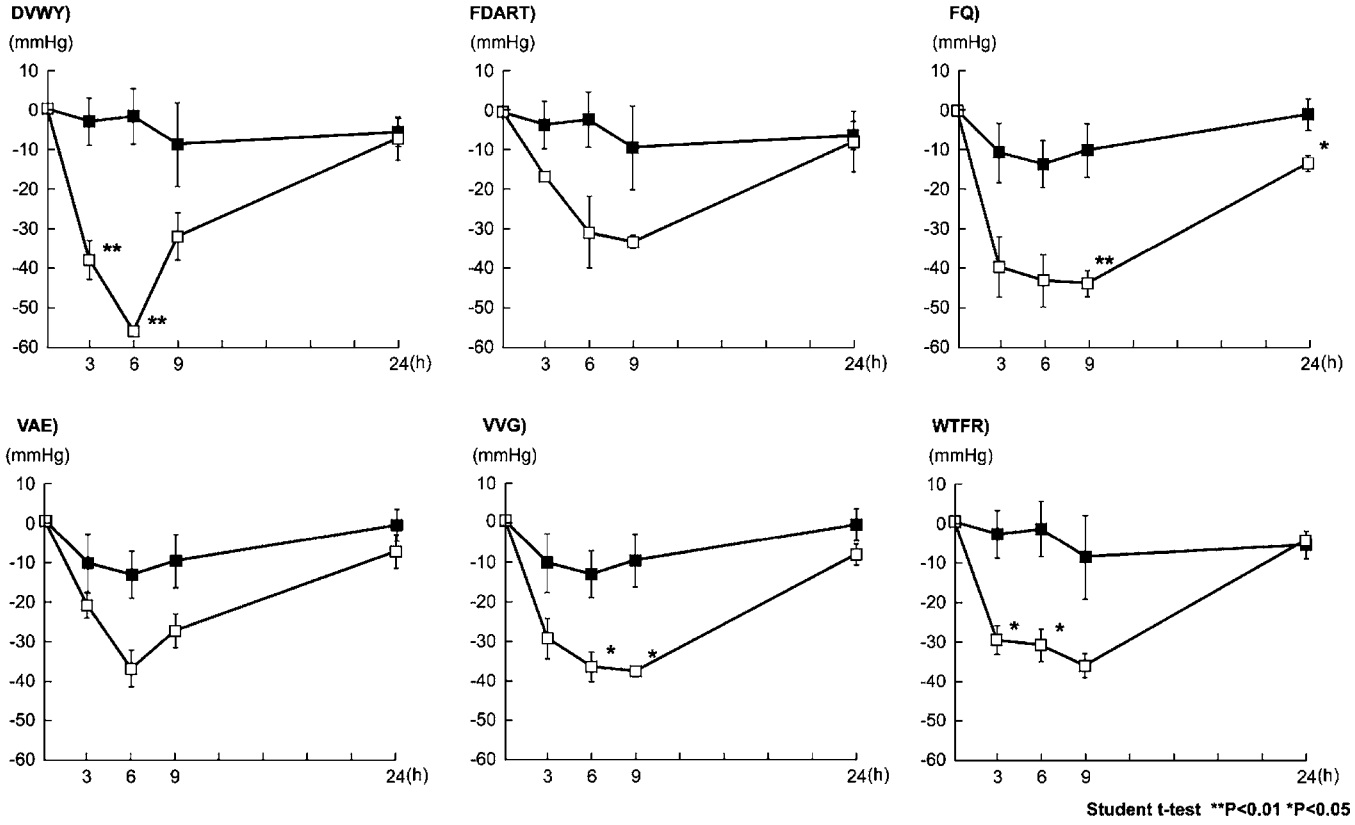

Figure 6. Changes in systolic blood pressure (SBP) of 13-week-old spontaneously hypertensive rats (SHRs) after a single oral administration of identified peptides at a dose of 0.10 mg/kg BW. □, identified peptides ($n = 5$); ■, purified water (control) ($n = 5$). Each point and bar represents the means ± SE. *$p < 0.05$; **$p < 0.01$, versus the control group at each time point as evaluated by Student's t-test.

could be partly responsible for the BPL effect of neo-FBS. Recently, we reported that the contents of rutin and its related phenol, isoorientin, which are well-known functional compounds in buckwheat, were significantly decreased through the lactic fermentation of BS.[12] Neo-FBS containing lower amounts of phenols was much more potent than BS, suggesting that the phenols are insignificant BPL compounds in neo-FBS. The content of a potent ACE inhibitor in buckwheat, 2″-hydroxynicotianamine, has been reported to be 3.32 times higher in FBS than in buckwheat green juice.[20] However, the *in vivo* BPL effect of the compound is not clear, making it difficult to evaluate its contribution to the BPL action of neo-FBS.

We investigated the capacity of the lactic fermentation process to induce the production of BPL compounds. In the results, six peptides (DVWY, FDART, FQ, VAE, VVG, and WTFR) and tyrosine were identified as candidate compounds from the fractionated peaks increased by lactic fermentation of BS. In another study, we reported that tripeptide GHG was identified in FBS.[21] This peptide was found in neo-FBS, but it had weak BPL activity (data not shown). There were no matches for the peptides DVWY, VAE, and WTFR in the SciFinder databases (https://scifinder.cas.org/)[22] and the Protein Research Foundation database (http://www.prf.or.jp/seqdb-e.html).[23] We also conducted a search for sequence alignments of matched regions by patterns and profiles using the UniProtKB/Swiss-Prot database (http://www.ebi.ac.uk/uniprot/)[24] of proteins contained in buckwheat. Sequences of FQ, VAE, VVG, and WTFR corresponded to residues 148−149 of ribulose bisphosphate carboxylase/oxygenase (Rubisco) proteins, residues 437−439 of ATP synthase CF1 beta subunit, residues 437−439 of hypothetical chloroplast RF1, and residues 91−94 of nucleocapsid protein, respectively. These are general plant proteins that are abundant in plant bodies as well as seeds.[25−28] In fact, previous reports have identified BPL peptides from chloroplast proteins and Rubisco proteins of spinach.[25,29] These data support that the identified peptides were generated by lactic fermentation from protein in BS. On the other hand, in this database,[24] the sequence of DVWY or FDART was not found in known buckwheat proteins. We speculate that sequences of these peptides could be from unknown proteins expressing in BS only or metabolites of the lactic acid bacteria.

The identification of newly produced peptides in neo-FBS suggested that they may be responsible for the dramatically higher BPL potency of neo-FBS compared to BS. Therefore, each peptide was synthesized to ensure purity and tested in SHRs using a single oral dose of 0.10 mg/kg BW. Comparative analysis revealed that all six peptides exhibited BPL activity in this model of hypertension, generating 30−60 mmHg reductions in SBP and/or DBP. The peptides showing the most consistent results for these two blood pressure parameters were DVWY, FQ, and VVG. Collectively, these studies suggest that the lactic fermentation process we developed for BS in our laboratory is responsible for the synthesis of new potent antihypertensive peptides.

The present study shows that lactic-fermented BS (neo-FBS) had higher quantities of known BPL compounds, tyrosine and GABA, as well as the six novel peptides. All identified peptides had significant BPL effects in SHRs, and their effects have never been reported before. Three of the peptides, DVWY, VAE, and WTFR, were considered to have novel sequences. We conclude that the new BPL peptides produced by lactic fermentation of

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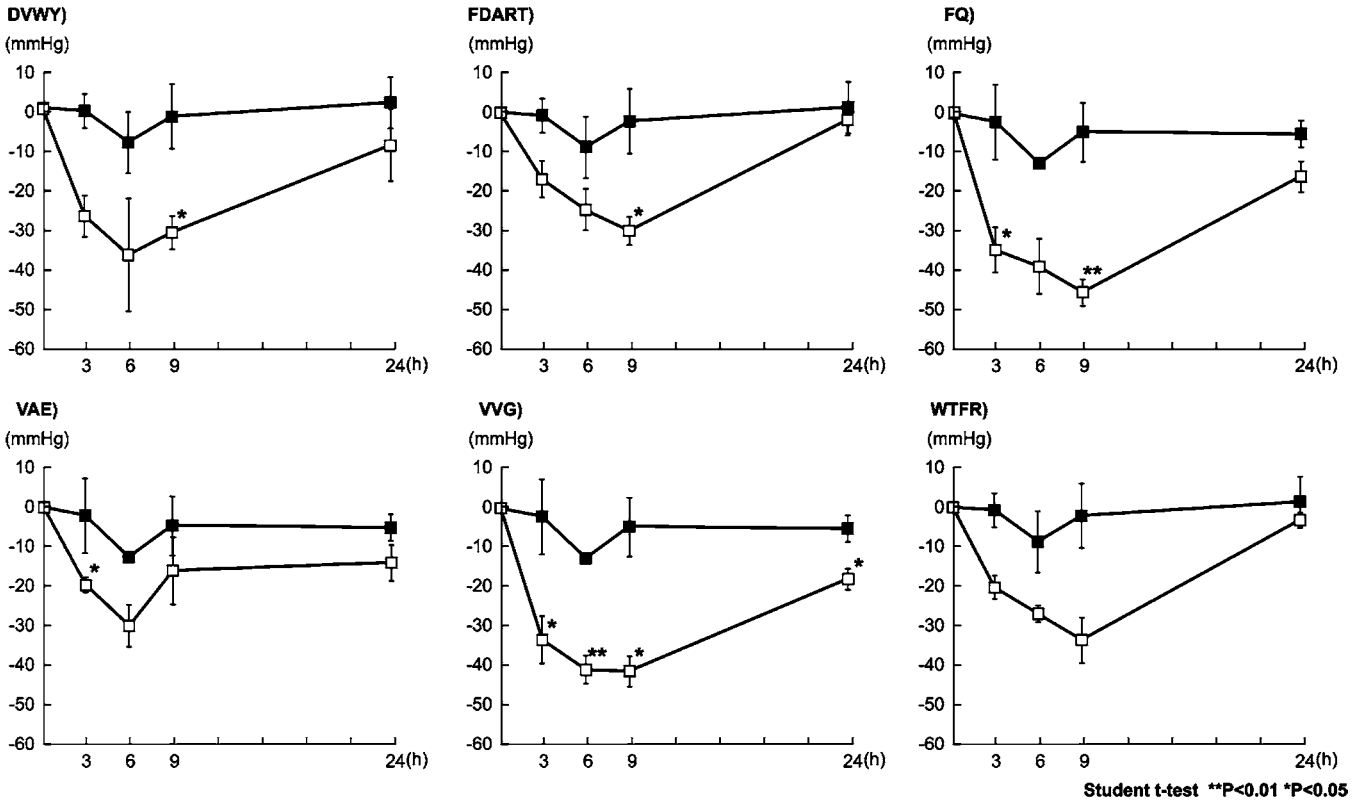

Figure 7. Changes in diastolic blood pressure (DBP) of 13-week-old spontaneously hypertensive rats (SHRs) after a single oral administration of identified peptides at a dose of 0.10 mg/kg BW. □, identified peptides ($n = 5$); ■, purified water (control) ($n = 5$). Each point and bar represents the means ± SE. *$p < 0.05$; **$p < 0.01$, versus the control group at each time point as evaluated by Student's *t*-test.

BS as well as the elevated existing BPL compounds are responsible for the potent BPL effects of neo-FBS. Studies are currently under way to evaluate the safety and efficacy of these compounds for chemopreventive and pharmaceutical applications.

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Funding

This work was supported in part by grants from a program from the Feasibility Study Stage, the Japan Science and Technology Agency (JST), the Ministry of Education, Science, Sports and Culture, Japan.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Saladcosmo Co., Ltd. for providing buckwheat sprouts.

ABBREVIATIONS USED

ACE, angiotensin I-converting enzyme; BPL, blood pressure lowering; Boc, *tertiary*-butoxy carbonyl; BS, buckwheat sprouts; BW, body weight; Bzl, benzyl; CHCA, α-cyano-4-hydroxycinnamic acid; DBP, diastolic blood pressure; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; DMTMM, 4-(4,6-dimethoxy-1,3,5-triazine-2-yl)-4-methylmorpholinium chloride; DW, dry weight; ESI, electrospray ionization; FBS, fermented buckwheat sprouts; neo-FBS, neo-fermented buckwheat sprouts; Fmoc, 9-fluorenylmethyloxycarbonyl; GABA, γ-aminobutyric acid; HBTU, *O*-(benzotriazol-l-y1)-1,1,3,3-tetramethyluronium hexafluorophosphate; HCl, hydrochloric acid; HOBt, *N*-hydroxybenzotriazole; MeOH, methanol; NaCl, sodium chloride; NaOCl, sodium hypochlorite; NaOH, sodium hydroxide; Na_2SO_4, sodium sulfate; nIPE, *n*-isopropyl ether; NMP, *N*-methylpyrrolidone; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PITC, phenylisothiocyanate; PPD, piperidine; PTC, phenylthiocarbamyl; Rubisco, ribulose bisphosphate carboxylase/oxygenase; SBP, systolic blood pressure; SE, standard error; SHRs, spontaneously hypertensive rats; S/N, signal to noise; *t*Bu, *tertiary*-butyl; TFA, trifluoroacetic acid; TIPS, triisopropylsilane

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