*Highlights (for review)

Highlights

- Neo-FBS is a lactic fermentation product of buckwheat sprouts.
- Neo-FBS demonstrated excellent blood-pressure-lowering (BPL) effects in SHRs.
- A single dose of 0.010 mg/kg of neo-FBS caused significant BPL.
- Neo-FBS inhibited ACE activity *in vivo* and caused arterial relaxation *ex vivo*.
- It was concluded that both effects were responsible for the excellent BPL effect.

*Manuscript
Click here to view linked References

Blood-pressure-lowering Effect of Fermented Buckwheat Sprouts in Spontaneously Hypertensive Rats

Kozo Nakamura[1,2,3]***, Kyoko Naramoto**[2]**, Masahiro Koyama**[3]

[1]*Department of Bioscience Biotechnology, Faculty of Agriculture, Shinshu University, Minamiminowa, Nagano 399-4598, Japan*

[2]*Department of Bioscience and Biotechnology, Graduate School of Agriculture, Shinshu University, Minamiminowa, Nagano 399-4598, Japan*

[3]*Department of Bioscience and Food Production Science, Interdisciplinary Graduate School of Science and Technology, Shinshu University, Minamiminowa, Nagano 399-4598, Japan*

*** Corresponding author:**

Dr. Kozo Nakamura

Faculty of Agriculture, Shinshu University

8304 Minamiminowa, Nagano 399-4598, Japan

Tel/Fax: +81-265-77-1638

Email: knakamu@shinshu-u.ac.jp

Running title: Fermented buckwheat sprouts lower BP

Abbreviations:

ACE:	Angiotensin I-converting enzyme
ANOVA:	Analysis of variance
BPL:	Blood-pressure-lowering
BS:	Buckwheat sprouts

BW:	Body weight
DBP:	Diastolic blood pressure
DW:	Dry weight
FBS:	Fermented buckwheat sprouts
neo-FBS:	Neo-fermented buckwheat sprouts
SBP:	Systolic blood pressure
SHRs:	Spontaneously hypertensive rats
TC:	Threshold concentration

Abstract

A practical antihypertensive food, neo-fermented buckwheat sprouts (neo-FBS), was produced from buckwheat sprouts by lactic fermentation. The neo-FBS preparation gave a 12.7 times better yield and had a 10 times more potent blood-pressure-lowering (BPL) effect than conventionally prepared products. Neo-FBS decreased both systolic and diastolic blood pressure in spontaneously hypertensive rats (SHRs) at a dose of 0.010 mg/kg, an effect comparable to 1.0 mg/kg captopril, an anti-hypertensive drug. Orally administered neo-FBS (10 mg/kg) significantly decreased angiotensin I-converting enzyme (ACE) activity in the lung, thoracic aorta, heart, kidney, and liver of SHRs. Neo-FBS had a detectable relaxing effect on a phenylephrine-precontracted thoracic aorta in SHRs at 0.5 μg/mL and the EC_{50} value was 8.3 ± 1.4 μg/mL. The ACE inhibition and vasorelaxation activities were found to be responsible for the excellent BPL effect of neo-FBS. As SHR is a standard model for human hypertension, neo-FBS may also have BPL effects in human patients.

Keywords: Antihypertension, vasorelaxation, fermented buckwheat sprouts, blood pressure

1. Introduction

Hypertension is a major risk factor for cardiovascular disease and stroke (Lloyd-Jones et al., 2010), thus prevention of hypertension is important in reducing the risk of these debilitating ailments. There is currently increasing interest in the identification of antihypertensive foods because they are expected to prevent hypertension with lower side-effects than antihypertensive drugs (Chen et al., 2009). Buckwheat (*Fagopyrum esculentum*), from the family *Polygonaceae*, is a potential antihypertensive food, and a significant number of reports concerning its blood pressure lowering (BPL) effects have been published (Guang & Phillips, 2009; Krkošková & Mrázová, 2005; Li & Zhang, 2001). Buckwheat seeds are a commonly eaten food in Asia and Western countries in the form of groats, flour, and noodles. The leaves and stems of the buckwheat plant are also edible and have been used as a traditional medicine in eastern Asia. The antihypertensive actions of buckwheat and its ingredients have been reported in a single oral administration test in rats, an *in vitro* angiotensin I-converting enzyme (ACE) inhibitory assay, and in the vasorelaxation of aortic preparations (Aoyagi, 2006; Fu et al., 2005; Fusi et al., 2003; Kim et al., 2009; Li et al., 2002; Matsui et al., 2010; Nakamura et al., 2005, 2008; Ushida et al., 2008). The most well-known functional compound in buckwheat, rutin, and the related phenol compounds orientin and isoorientin, are reported to possess vasorelaxant effects on isolated rat thoracic aorta (Fu et al., 2005; Fusi et al., 2003). The potent ACE inhibitor 2″-hydroxynicotianamine is also present in buckwheat seeds and plants (Aoyagi, 2006). The osbeckic acid in tartary buckwheat extract is a newly discovered non-rutin vasorelaxation factor (Matsui et al., 2010; Ushida et al., 2008). Buckwheat flour hydrolysate has ACE inhibitory activity *in vitro*, and several ACE inhibitory peptides have been identified from this preparation (Li et al., 2002). Recently, it has been reported that hot water extracts of buckwheat seeds and germinated buckwheat, which are buckwheat seeds that have just begun to sprout, have an antihypertensive effect in spontaneously hypertensive rats (SHRs) treated at a dose of 600 mg/kg/day for 5 weeks (Kim et al., 2009).

Despite all of these potential activities, however, the practical antihypertensive effect has not yet been demonstrated in buckwheat consumed as food. In 2005, we reported a preparation of fermented buckwheat sprouts (FBS) made from hydroponically-cultivated buckwheat plants, and in 2008 we also reported the *in*

vivo BPL effects and *in vitro* ACE inhibitory activity of the FBS (Nakamura et al., 2005, 2008). In this work, we report the production of newly-generated FBS (neo-FBS) that has excellent BPL properties and contains phenols, which are major functional ingredients in buckwheat. The present study describes the new preparation method, and the BPL and antihypertensive effects of neo-FBS in SHRs compared to the anti-hypertensive drug captopril. In addition, the mechanism of action of neo-FBS investigated in the aorta rings and tissue samples of SHRs is reported for the first time. At the end, we discuss the contribution to BPL of phenols in neo-FBS based on quantitative analyses of gross phenols and the five kinds of buckwheat phenols, including rutin, in raw buckwheat sprouts (BS) and neo-FBS.

2. Materials and Methods

2.1 Chemicals

Sodium hypochlorite (NaOCl), captopril, phenylephrine hydrochloride (PE), and acetylcholine chloride (ACh) were purchased from Wako Pure Chemical Industries (Osaka, Japan). ACE (EC 3.4.15.1) from rabbit lung, Krebs-Henseleit buffer, Folin–Ciocalteu phenol reagent, and sodium carbonate were from Sigma-Aldrich (St. Louis, MO, USA). Hippuryl-L-histydyl-L-leucine (Hip-His-Leu) as a substrate for ACE was from the Peptide Institute (Osaka, Japan). HPLC-grade acetonitrile was from Kanto Chemical Co., Inc. (Tokyo, Japan). Other reagents used in the experimental procedures were of analytical grade and used without further purification. Purified water was obtained from a Sartorius purification system (Sartorius AG, Gottingen, Germany).

2.2 Experimental animals

All of the experiments in the present study were carried out using 13-week-old male SHRs weighing 300 to 350 g. Twelve-week-old SHRs (Charles River Laboratories Japan Inc., Kanagawa, Japan) were maintained in individual cages (1 rat/cage) in a temperature-controlled room (23 ± 2°C) with a 12-h light/dark cycle (lights on at 8 AM) and had free access to tap water and the commercial powder feed (MF; Charles River Laboratories Japan Inc.) for 1 week until the experiment was performed. Experimental and

surgical procedures were performed with the approval of the Animal Care Committee of the Faculty of Agriculture of Shinshu University (approved number: 190104).

2.3 Plant material

To produce neo-FBS, we used BS manufactured by a plant factory as raw materials. BS were cultivated and provided by SALADCOSMO CO. Ltd. (Gifu, Japan). Buckwheat seeds were surface-sterilized with ozone and immersed in water at 32–33° C for 5.5 h. The seeds were planted in soaking-wet urethane foam for 24 h in the dark at 23°C and then grown in a glasshouse with culture fluid under weak natural sunlight for about 10 days. The culture fluid was maintained at 18–20° C and at pH 5.5–6.0 and was sprayed onto the seeds for 1 min every 4 h. When the plants were around 15 cm tall, they were harvested and the roots and seed hulls were removed. For each preparation of neo-FBS, freshly harvested BS was used. A part of harvested BS (25.0 g) was sterilized using NaOCl solutions and was subsequently ground with a juicer. Ground BS was squeezed and centrifuged at 3600 *g* for 30 min at 5° C (Centrifuge 5810 R; Eppendorf Co. Ltd., Tokyo, Japan). The supernatant was lyophilized using a freeze dryer (EYELA FDU-2000; Tokyo Rikakikai Co. Ltd., Tokyo, Japan), and 274.8 mg of BS dry powder was obtained in the yield of 1.10% and used for subsequent analyses as lyophilized BS.

2.4 Preparation of neo-FBS

Freshly harvested BS (10.0 kg) was sterilized by soaking in a 100 ppm effective chlorine concentration solution of NaOCl (20 L) for 10 min. After rinsing with water, the sterilized BS were cut into 2-cm pieces and ground with a commercially available juicer. A starter solution of *Lactobacillus plantarum* KT (Biotech Japan, Agano, Japan) was added to the ground BS in a volume of 25 mL/kg of BS and stored in a tightly covered container (15 L). After expelling the air in the container, the fermentation process was started under an inert gas (nitrogen) atmosphere. After 2 weeks culture at room temperature, 8.1 kg of neo-FBS product was obtained. The product was squeezed and centrifuged at 3600 *g* for 30 min at 5° C (Centrifuge 5810 R) to obtain a pink-coloured supernatant (6.80 kg). The supernatant was lyophilized (EYELA FDU-2000), and

137.4 g of red powder was obtained.

2.5 Antihypertensive effect of a single oral dose of neo-FBS in SHRs

Animals were assigned to four groups such that the average blood pressure for all rats was the same in each group. After being deprived of food for 12 h, single oral administrations were given to the rats by gastric intubation. Aqueous solutions of the lyophilized neo-FBS were orally administered to rats at a dose of 0.10 (n = 10) or 0.010 (n = 6) mg/kg body weight (BW). The positive control group (n = 6) was administered aqueous solutions of captopril at a dose of 1.0 mg/kg BW, and the negative control group (n = 10) was administered purified water. Before and at 3, 6, 9, and 24 h after administration, the systolic blood pressure (SBP) and the diastolic blood pressure (DBP) of the rats were measured with a Softron BP98A tail cuff (Softron Co. Ltd., Tokyo, Japan). Before measurement, the rats were kept at 38° C for 10 min to make the pulsations of the tail artery detectable. Changes in SBP and DBP were calculated as the difference between the mean values of six measurements obtained before and after the administrations, and the results were expressed as the mean ± standard error (S.E.).

2.6 Determination of ACE activity

2.6.1 ACE inhibition activity

The ACE inhibition assay was performed according to the method of Horiuchi et al. (1982) with minor modifications. Three series of assay samples were prepared at 0.10, 0.20, and 0.40 mg/mL of lyophilized neo-FBS and 10, 20, and 40 ng/mL of captopril in purified water. Each 30 μL of sample solution was preincubated with Hip-His-Leu borate buffer (7.6 mM Hip-His-Leu, 0.10 M borate, and 0.60 M NaCl (pH 8.3)) at 37° C for 7 min. One hundred microliters of ACE (6.0 mU) were then added, and the mixture was incubated at 37° C for another 30 min. The reaction was stopped by the addition of 250 μL of 1 M HCl. The resulting hippuric acid was extracted with 1 mL of ethyl acetate, and the solution was centrifuged at 13,000 *g* for 5 min (Centrifuge 5415 R, Eppendorf). The 800 μL upper layer were transferred to a test tube and evaporated at room temperature for 1 h under vacuum. The hippuric acid was dissolved in 500 μL of purified

water and quantified by HPLC (LC-2010CHT; Shimadzu Co., Kyoto, Japan). Acetonitrile with 0.10% (v/v) TFA was used as the mobile phase and elution was performed with 0.10% (v/v) TFA in purified water. Separations were performed using a 150 mm × 4.6 mm ODS column (TSK-Gel ODS 120A; Tosoh Co., Tokyo, Japan) and chromatography was performed at 40° C with a flow rate of 1.0 mL/min, an injection volume of 20 μL, and detection at 228 nm. The linear calibration curve for hippuric acid was obtained in the concentration range of 5.0–50 μg/mL with a correlation coefficient of 0.9991; linearity was described by the equation $y = 27125x + 180977$. The amounts of liberated hippuric acid in the sample, sample control, blank, and blank control were calculated from the calibration curve. In the sample control, 1 M HCl was added before the sample solution; in the blank, purified water was used in place of the sample solution; and in the blank control, 1 M HCl was added before the purified water. The percent inhibition of ACE activity was calculated from the equation $[((B-Bc) - (S-Sc))/(B- Bc)] \times 100$, where S, Sc, B, and Bc are the contents of liberated hippuric acid in the test sample, the sample control, the blank, and the blank control, respectively. The concentration of the neo-FBS and captopril that inhibits 50% of ACE activity (IC_{50}) was determined by plotting the content of hippuric acid against the percent of ACE inhibition. All measurements were performed in triplicate with each concentration of samples and the results were expressed as the mean ± S.E.

2.6.2 Measurements of plasma and tissue ACE activity

Animals were assigned to three groups of six animals each, a neo-FBS administered group, a positive control group, and a negative control group. Neo-FBS dissolved in purified water was administered to SHRs at a dose of 10 mg/kg BW, the positive control group was administered captopril at a dose of 10 mg/kg BW, and the negative control group was administered purified water only. All administrations were performed as described in section *2.5*. Before the administration, the SBP and DBP of all test rats were measured by the tail-cuff method. At 6 h after the administration, the SBP and DBP of test rats in each group were measured again to confirm the BPL effect. Immediately after the blood pressure measurement, the rats were anesthetized with diethyl ether and then exsanguinated via the abdominal aorta. Blood samples from each SHR were collected into tubes containing lithium heparin as an anticoagulant and centrifuged at 3200 *g* for

20 min at 4° C to separate the plasma. The lungs, thoracic aortas, hearts, kidneys, and livers were immediately excised, weighed, and then frozen. Tissue samples for ACE activity assays were prepared as follows according to the previously described protocol (Horiuchi et al., 1982). After thawing, the chopped tissues were homogenized at 0° C in 50 mM Tris-HCl (pH 7.8) containing 30 mM KCl, 5 mM magnesium acetate, 0.25 M sucrose, and 0.5% Nonidet P-40 in a Potter homogenizer (Iuchi, Osaka, Japan) for 5 (lungs, heart, kidney, and liver) or 10 (thoracic aorta) min. The suspension was centrifuged at 12,000 *g* for 20 min at 4° C, and the supernatant was immediately used to measure ACE activity. Measurement of plasma and tissue ACE activities was carried out according to the method described in the previous section. The specific activity was expressed as the number of units per milligram of protein. The protein contents in the samples were determined by the bicinchoninic acid assay using the BCA Protein Assay Reagent Kit (Thermo Fisher Scientific K.K., Kanagawa, Japan).

2.7 SHR ring aorta assay

The impact of neo-FBS on vascular contractility of aortic rings in SHRs was determined by constructing dose-response curves. Six individual SHRs were anesthetized with diethyl ether and then sacrificed by the exsanguination described in section *2.6.2*. The thoracic aorta was immediately isolated, cleaned of fat and connective tissue in Krebs-Henseleit buffer solution, and cut into approximately 3–5 mm rings. The ring segments were suspended on stainless steel hooks in a 5 mL organ bath containing warmed (37° C) and oxygenated (O_2:CO_2, 19:1) Krebs-Henseleit solution. A basal tension of 1.5 g was used for the tissues and changes in basal tension were recorded by an isometric force transducer (UFER UM-203; Kishimoto Medical Instruments, Kyoto, Japan) connected to a micro-easy magnus system (UFER UC-05A; Kishimoto Medical Instruments). After equilibration for 60 min, the rings were stimulated with 0.30 μM PE (Sauvaget et al., 2010). The presence of endothelium was confirmed by relaxation with 100 μM Ach, and relaxation of at least 90% was considered evidence for the functional integrity of the endothelium. The stimulant agents were removed, and the tissues were washed twice with the Krebs solution. The initial basal tension was resumed and the vascular tissue was contracted again with 0.30 μM PE. After reaching

maximum contraction, the PE was washed out and the procedure was repeated once more for stabilization. After the final priming step, the vascular tissues were contracted with 0.30 μM PE, and when stable contraction was obtained, the neo-FBS test samples were added to the organ bath at concentrations ranging from 0.50 to 100 μg/mL. The relaxation of the vascular tissue (vasorelaxation) was expressed as the percentage of initial contraction baseline induced by PE. The results are expressed as the mean ± S.E. The EC_{50} value of the neo-FBS was calculated from the relaxation curve. At the end of each experiment, 100 μM papaverine was added to the organ bath to induce complete relaxation of the aortic rings. A similar test was performed using captopril in concentrations ranging from 50 to 1000 μg/mL.

2.8 Quantification of flavonoids and total phenols in BS and neo-FBS

2.8.1 Flavonoids

All HPLC analyses were performed with a Prominence HPLC system (Shimadzu Co., Kyoto, Japan). The flavonoids in BS and neo-FBS were analyzed as follows. Three milligrams each of lyophilized BS and neo-FBS were dissolved in 1 mL of 50% methanol–water. Separation was performed at 40° C using a CHEMCOBOND 5-ODS-W reversed phase column (4.6 × 150 mm; ChemcoPlus Scientific Co., Ltd. Osaka, Japan). Gradient elution was performed at a flow rate of 0.8 mL/min using two mobile phases, 0.1% formic acid in purified water (solvent A) and 0.1% formic acid in acetonitrile (solvent B): 0–2 min, gradient 0–5% solvent B; 2–12 min, gradient 5–8% solvent B; 12–16 min, gradient 8–10% solvent B; 16–18 min, gradient 10–14% solvent B; 18–20 min, gradient 14–15% solvent B; 20–50 min, isocratic 15% solvent B. Ultra violet (UV) detection was performed at 280 nm and the injection volume was 10 μL. Flavonoid contents were standardized against rutin and expressed as rutin equivalents (mg/g dry weight (DW)). Standard solutions were prepared in 50% methanol–water and were analyzed in a manner similar to that for BS and neo-FBS samples. A linear calibration curve for rutin was obtained in the concentration range of 0.125–1.0 mg/mL, with a correlation coefficient of 0.9999; linearity was described by the equation y = 107x + 81639. All measurements were performed in triplicate and the results were expressed as mean ± S.E.

2.8.2 Total phenols

Total phenol concentrations were measured using the Folin–Ciocalteu assay (Julkunen-Tiitto et al., 1985). Lyophilized BS and neo-FBS were dissolved in purified water to a concentration of 0.5 mg/mL. To a 10-mL test tube, 1.0 mL of BS or neo-FBS samples and 1.0 mL of 1 M Folin–Ciocalteu reagent was added. After the contents were stirred for 2 min, 1 mL of an aqueous 10% sodium carbonate solution was added to the mixture. Solutions were stirred and allowed to stand at room temperature for 30 min. Total phenol concentrations were determined using a UV-2500PC spectrophotometer (Shimadzu Co., Kyoto, Japan) at 700 nm. Total phenol contents were standardized against gallic acid and were expressed as gallic acid equivalents (mg/g DW). The linear calibration curve for gallic acid was obtained in the concentration range of 0.01–0.04 mg/mL with a correlation coefficient of 0.9996, and linearity was described by the equation $y = 33.14x + 0.146$. Ascorbic acid correction was not performed because ascorbic acid was not contained in BS and neo-FBS. All measurements were performed in triplicate with each concentration of the samples, and the results were expressed as mean ± S.E.

2.9 Statistical and data analysis

All results are expressed as the mean ± S.E. Changes in plasma and tissue ACE activity were analyzed by one-way analysis of variance (ANOVA) followed by Student’s *t*-test. The unpaired Student's *t*-test was used for statistical analyses of BPL and its vasorelaxing effect, and the content of total phenols and flavonoids. A p value less than 0.05 was considered statistically significant.

3. Results

3.1 Production of neo-FBS

The neo-FBS in this report was produced by improvements to a method that we had previously reported (Nakamura et al. 2005). In the conventional FBS preparation, the starting material was raw juice squeezed from BS that was fermented with naturally occurring lactobacillus and/or yeast in a refrigerator. The fermenting mixture was frozen at about –25° C to halt the development of septic organisms. In the new

method, whole crushed BS was used as the starting material and the freezing step was omitted. By adding a *lactobacillus* starter culture before fermentation, the lactate fermentation proceeded under anaerobic conditions and the pH of the fermenting mixture dropped from 5.4 to 3.8 in 24 h at room temperature. The low pH and anaerobic conditions suppressed the growth of *E. coli* and fungus at room temperature. The liquid product was then squeezed from the neo-FBS when the fermentation was finished. Based on the fresh weight of starting materials, the yields of liquid and lyophilized product with the new method were 68.2 and 1.4%, respectively, which were 7.5 and 12.7 times those from the conventional method using the same starting material, BS (9.1 and 0.11%, respectively).

3.2 Blood-pressure-lowering effect

Figure 1 shows the BPL effects of neo-FBS after a single oral administration to 13-week-old male SHRs at doses of 0.010 and 0.10 mg/kg BW together with positive and negative control groups. At a dose of 0.10 mg/kg BW, both SBP and DBP were significantly decreased compared with the negative control ($p < 0.010$) after a 3 h after administration. The BPL effects continued with time and were maintained for over 9 h before returning to baseline levels at 24 h. The maximum effects were observed 9 h after the single oral administration, and the means of SBP and DBP had decreased by 31.3 and 23.9 mm Hg compared to the negative control group, respectively. Even at a 10 times lower dose (0.010 mg/kg), the BPL effect of the neo-FBS on SHRs was significant, and the time-dependent changes to both SBP and DBP were similar to those of rats that had received the higher dose. Significant decreases in SBP and DBP were seen 3 h after administration, and the SBP and DBP values were 22.2 and 14.2 mm Hg, respectively, compared to a negative control group at 9 h post-administration. In the captopril-administered group, only the SBP was significantly decreased compared with the negative control. There were no significant differences between the two neo-FBS doses when compared to each other, and the 0.10 mg/kg group showed a significant reduction in BPL compared to the positive control.

3.3 ACE inhibition activity

In vitro ACE inhibition by the neo-FBS was determined by the HPLC method (Horiuchi et al., 1982). The inhibitory activity of the neo-FBS was expressed as the IC_{50} value (the concentration of neo-FBS required to inhibit 50% of the ACE activity). An IC_{50} value of 0.22 ± 0.01 mg/mL for the neo-FBS was similar to the value 0.24 ± 0.03 mg/mL obtained for material prepared by the conventional method. The IC_{50} value of captopril was 20 ± 1.2 ng/mL in our assay.

The *in vivo* ACE activities of body tissues (plasma, lungs, thoracic aorta, heart, kidney, and liver) from test groups at 6 h after administration were also determined in a similar manner. As shown in Fig. 2, at the 10 mg/kg dose of neo-FBS, the mean values of ACE activities of plasma, lungs, thoracic aorta, heart, kidney, and liver were 82.5 mU/mL, and 54.9, 19.2, 0.15, 0.72, and 0.021 mU/mg of protein, respectively. On the other hand, those of the negative control group administered purified water only were 78.1 mU/mL and 70.2, 30.2, 0.40, 0.86, and 0.054 mU/mg of protein, respectively. The neo-FBS administration significantly lowered ACE activity in lungs, thoracic aorta, heart, kidney, and liver tissues from SHRs, but no significant reduction in activity was seen with the plasma sample. In the positive control group receiving 10 mg/kg of captopril, ACE activities in the plasma, lungs, thoracic aorta, heart, kidney, and liver were found to be 60.5 mU/mL and 56.1, 24.6, 0.36, 0.33, and 0.014 mU/mg of protein, respectively. The oral administration of captopril produced a significant reduction in ACE activity in lungs, thoracic aorta, kidney, and liver compared to the negative control group. In plasma and heart tissues, ACE activity was reduced but the amounts were not statistically significant. ACE inhibition in lung and liver tissues from neo-FBS-treated rats was the same as that for captopril-treated rats, while in the thoracic aorta and heart tissues, ACE activity was significantly more inhibited in the neo-FBS group compared to the captopril group. In the plasma and kidney, ACE activity was significantly lowered in the captopril group compared to the neo-FBS group. Just before administering the anaesthesia, the mean values of SBP and DBP were lower by 31.2 and 34.6 mm Hg in the neo-FBS group, and 31.6 and 21.9 mm Hg in the captopril group compared with the negative control group. The decreases in SBP and DBP in the neo-FBS group and SBP in the captopril group were significant compared to the negative control group.

3.4 Vasorelaxing effect of the neo-FBS

Figure 3 shows a typical relaxation profile upon addition of neo-FBS to endothelium-intact thoracic aorta rings that had been pre-contracted with 0.30 μM PE. The neo-FBS had a dose-dependent vasorelaxation effect on the blood vessel rings. Figure 4 shows a relaxation curve made by plotting the mean values of six individual tests of endothelium-intact thoracic aorta rings. The threshold concentration (TC) for the neo-FBS to induce significant vasorelaxation was only 0.50 μg/mL, and 50 μg/mL produced a maximum relaxation effect of 82.8 ± 3.7%. The effective concentration causing 50% relaxation (EC_{50}) of PE-precontracted thoracic aorta was calculated as 8.3 ± 1.4 μg/mL. When 100 μM papaverine was added to the organ bath at the end of the experiment, 3.2 ± 1.8% relaxation of the aorta rings was observed. On the other hand, the percentage of vasorelaxation caused by captopril was only 12.0 ± 5.3% even at concentrations as high as 1000 μg/mL.

3.5 Content of phenols in BS and neo-FBS

To confirm participation of phenols present in BS in the BPL action of neo-FBS, we determined the contents of these compounds in BS and neo-FBS. Table 1 shows the quantities of total phenols and five kinds of major flavonoids in BS and neo-FBS. Total phenols in BS and neo-FBS were determined by the Folin-Ciocalteau method and the results were expressed as gallic acid equivalents. The amounts of total phenols were 40.5 ± 0.3 and 43.0 ± 1.2 mg/g DW gallic acid equivalents, respectively. The contents of orientin, isoorientin, vitexin, isovitexin, and rutin were determined by the method previously reported (Koyama et al., 2010) and were expressed as rutin equivalents. The results showed that the contents of isoorientin and rutin were significantly decreased in neo-FBS, but the contents of the other flavonoids remained the same. As a result, the total phenol content was the same in both preparations, but the amounts of rutin and isoorientin in neo-FBS were lower than those in BS.

Discussion

In this report, we demonstrate the excellent BPL effect of a novel lactic acid fermentation preparation of

fermented buckwheat sprouts that we have termed neo-FBS. The BPL effect of the raw BS was much weaker than that of the neo-FBS, and the starter culture of *Lactobacillus plantarum* did not have any BPL effect (data not shown). The neo-FBS production process was basically the same as the traditional production method of unsalted, pickled vegetables except for the fermentation temperature (Watanabe et al., 2009). The neo-FBS was produced at room temperature, while traditional Japanese pickled vegetables are manufactured at low temperatures to suppress the growth of septic organisms. By adding a starter culture of *Lactobacillus* to easily consumed, crushed BS, the lactic acid fermentation process started quickly and lowered the pH fast enough to prevent contaminant growth even at room temperature. Because buckwheat sprouts are a commercially available food with a long history of use, and neo-FBS is simply the supernatant from traditionally pickled vegetables (it is sour but can be drunk straight), neo-FBS is expected to be safe for human consumption.

Our lab has previously shown a significant decrease in both SBP and DBP after a single oral administration of 0.10 mg/kg BW of conventional FBS to 13-week-old SHRs (Nakamura et al., 2008). We have now shown that neo-FBS significantly decreases both SBP and DBP even at a dose as low as 0.010 mg/kg BW. Other food-based products with a reported BPL effect include hydrolysates or enriched food extracts such as peptides and phenols. The minimum effective doses of hydrolysates of krill, egg white, spinach leaf protein, and dried bonito were 1.0, 150, 250, and 500 mg/kg BW, respectively (Fujita et al., 1995; Hatanaka et al. 2009; Miguel et al., 2005; Yang et al., 2004) and those of aqueous ethanol extracts of sardine hydrolysate and Brazilian propolis, and a hot-water extract of green coffee beans were 10, 50, and 714 mg/kg BW, respectively (Mishima et al., 2005; Seki et al, 1999; Suzuki et al., 2006). BPL tests with single oral administrations of whole food products in SHRs are rare in the literature. At a dose of 5 mL/kg BW, lactic acid fermentation of 9% (w/w) skim milk significantly lowered the SBP of 20-week-old SHRs compared to a control group receiving the same volume of unfermented milk (Nakamura et al., 1995). A 150-mg/kg dose of “red mold dioscorea” significantly decreased SBP and DBP in SHRs 8 h after administration (Wu et al., 2009). From these studies, the effective range for food materials to exert a BPL effect was estimated to be from 1.0 to 500 mg/kg BW in a single oral administration to SHRs. In comparison,

the minimum effective dose of neo-FBS was 0.010 mg/kg BW and the BPL effect was greater than that previously reported for any other food product. As shown in Fig. 1, both the doses of neo-FBS, 0.10 and 0.010 mg/kg BW, were more efficient than the common antihypertensive drug, captopril. A dose of 1.0 mg/kg BW of captopril was enough to reduce the blood pressure in SHR (Sweet et al., 1981). Comparing the BPL effects of neo-FBS and captopril directly provides evidence for the excellence and supported therapeutic application of neo-FBS.

In this study, we have shown that *in vivo* ACE inhibition and vasorelaxation effects were the contributing factors behind the excellent BPL effect of neo-FBS. The IC_{50} value of neo-FBS was 0.22 mg/mL in the *in vitro* ACE inhibition assay, while *in vitro* ACE inhibition activities of various fermented foods have IC_{50} values ranging from 0.16 to 85.6 mg/mL (Okamoto et al., 1995). Among them, cheeses show superior ACE inhibition *in vitro* with IC_{50} values in the range of 0.16 to 0.26 mg/mL. Other fermented foods with ACE inhibitory activity include fermented soybean seasoning (IC_{50}, 0.45 mg/mL), fermented soybean paste (IC_{50}, 0.28 mg/mL), and fermented blue mussel sauce (IC_{50}, 1.01 mg/mL) (Je et al., 2005; Nakahara et al., 2010; Shin et al., 2001). Comparing these IC_{50} values, the *in vitro* ACE inhibitory activity of neo-FBS is found to be moderate compared to other antihypertensive food products. The *in vivo* ACE inhibition of neo-FBS, however, is significantly greater than its *in vitro* inhibition and occurs at lower doses. Oral administration of neo-FBS (10 mg/kg BW) significantly decreased ACE activities in the lung, thoracic aorta, heart, kidney, and liver of SHRs by 21.9, 36.4, 61.6, 16.1 and 60.6% compared to negative control (water administration), respectively. Although these tissue-dependent differences in ACE activities are similar to those described in the literature, the maximal depressive effect on ACE activity was found in heart (Nakamura et al., 1996), which should be a unique advantage of neo-FBS. The blood pressure of test SHRs was significantly decreased, and these tissue ACE inhibitions must contribute to the BPL effects of the neo-FBS. Upon oral administration of captopril (10 mg/kg BW), ACE activity was decreased in the plasma, lungs, thoracic aorta, heart, kidney, and liver by 22.6, 20.1, 18.7, 10.4, 61.6 and 74.7%, respectively. The difference was significant compared to controls in all of the tissues, except for the plasma and heart, and these tissue-specific activities are similar to previously reported data (Miguel et al., 2007). Thus, neo-FBS is

distinguished by its ability to inhibit ACE activity in the thoracic aorta and heart to a greater extent than captopril.

The *in vitro* ACE inhibitory activity of captopril (IC_{50}, 20 ng/mL) was much stronger than that of the neo-FBS (0.22 mg/mL). However, our results suggest that the *in vivo* ACE inhibitory activity of neo-FBS is not significantly different from that of captopril at the same dose. Furthermore, as described in section *3.2*, the BPL effect of neo-FBS in SHRs was superior to that of captopril because neo-FBS demonstrated the same BPL effect as captopril but at a thousand times lower dose. Thus, the *in vivo* data does not correlate with the *in vitro* data for ACE inhibition. It has been reported that other food-derived ACE inhibitory peptides possess higher BPL effects than would be expected from their *in vitro* ACE inhibitory activities (Vermeirssen et al., 2004; Rafik et al., 2012), and some of these inhibitory peptides have higher affinities for certain tissues and are more stable *in vivo* than captopril (Fujita & Yoshikawa 1999). These data indicate that the ACE inhibitory activities observed in *in vitro* experiments are not necessarily indicative of the compound's *in vivo* BPL effect. Therefore, it appears that neo-FBS is an efficient BPL food with high bioavailability and stability under physiological conditions, even though it's ACE inhibitory activity *in vitro* was moderate.

In addition to its ACE inhibition activity, neo-FBS has a vasorelaxing effect that contributes to its potent BPL effect in SHRs. The EC_{50} value was calculated to be 8.3 ± 1.4 μg/mL, and this value is low in comparison to other food products that have vasorelaxing properties. Polyphenol-rich cocoa powder provides around 9% vasorelaxation of methoxamine-precontracted thoracic aorta at doses as low as 1.0 ng/mL and has an EC_{50} value of 2.0 μg/mL (Quiñones et al., 2011). Methanol extract of *Hibiscus sabdariffa* L. calyces has vasodilatory effects on PE-precontracted thoracic SHR aorta from doses of 10 ng/mL, with an EC_{50} value of 150 μg/mL (Ajay et al., 2007), and chymotryptic digest of ovalbumin at a dose of 0.50 mg/mL causes vasodilation of PE-precontracted thoracic aorta (Matoba et al., 1999). Compared to data for other food products, neo-FBS appears to have potent vasorelaxant activity, and this is probably an important contributor to its excellent BPL effects in SHRs. ACE inhibition mostly suppresses SBP because ACE catalyzes both the production of the vasoconstrictor angiotensin II and the inactivation of the vasodilator bradykinin (Kouno et

al., 2005). Captopril's *in vivo* effect on SBP as opposed to DBP (Cienfuegos-Jovellanos et al., 2009; Miguel et al., 2006, 2009) is consistent with its effects on lowering the SBP in hypertensive patients (Tonkin & Wing, 1996).

Several studies have reported that phenolic compounds contained in plant body have bioactivities (Chandrasekara & Shahidi, 2011; Cheng et al., 2009; Li et al., 2009). Therefore, these potent BPL actions of neo-FBS are expected to be caused by an increase in the amount of BPL compounds, including phenols, in BS during the lactic fermentation. Rutin, orientin, and isoorientin are BPL compounds in buckwheat that have been described before (Fu et al., 2005; Fusi et al., 2003). Quantitative analysis of the buckwheat phenols in BS and neo-FBS showed that rutin and isoorientin were significantly decreased in neo-FBS but there was no significant change in the other flavonoids. The BPL effect of the raw BS was much weaker than that of the neo-FBS, as described above. These results indicate that the buckwheat phenols are probably not responsible for the BPL action of neo-FBS because neo-FBS containing smaller amounts of the phenols still caused much more potent reduction in blood pressure.

In this study, we have used SHRs and their tissues as a genetic model of hypertension (Su et al., 2004). The effects seen in the rats can be extrapolated to humans because the rat model exhibits a similar pathophysiology to humans, such as vascular endothelial dysfunction and sensitivity to oxidative stress (Russell & Proctor, 2006). Kleiber (1961) reported that basal metabolic rates of terrestrial mammals are proportional to 3/4th the power of their body weight. When it is assumed that the body weight of an adult human and a 13-week-old SHR are 60 and 0.30 kg, respectively, a human with 200 times the weight of a SHR has a 53.2 times greater metabolic rate than the SHR. Thus, it is expected that a single oral dose of 160 μg lyophilized FBS or 7.8 mL liquid FBS in an individual weighing 60 kg, under similar conditions to our experiment, should lead to a decrease in blood pressure; i.e., if it is administered to a slight to moderately hypertensive individual after he/she has been deprived of food for 12 h with only water intake for 9 h after the administration.

Based on our current data, we conclude that the dual effects of ACE inhibition and vasodilation were responsible for the neo-FBS lowering the blood pressure of the hypertensive rats. ACE inhibition is the most

popular BPL mechanism used for commercially available antihypertensive foods (Inoue et al., 2003; Mäkinen et al., 2012; Okamoto et al., 1995), and vasodilation has been reported as one of the important BPL mechanisms in these foods (Jäkälä et al., 2009; Matoba et al., 1999). These BPL actions are caused by active food ingredients in neo-FBS. It is expected that neo-FBS contains BPL products produce by the lactic fermentation of BS, including peptides and γ-aminobutyric acid (GABA) (Inoue et al., 2003). It is assumed that these active compounds in neo-FBS could cause ACE inhibition and vasodilation *in vivo* and result in lowering of blood pressure. We are currently conducting studies to identify BPL compounds in neo-FBS to demonstrate their role in the BPL action of neo-FBS.

References

Ajay, M., Chai, H. J., Mustafa, A. M., Gilani, A. H., & Mustafa, M. R. (2007). Mechanisms of the anti-hypertensive effect of *Hibiscus sabdariffa* L. calyces. *Journal of Ethnopharmacology*, 109, 388-393.

Aoyagi, Y. (2006). An angiotensin-I converting enzyme inhibitor from buckwheat (*Fagopyrum esculentum* Moench) flour. *Phytochemistry*, 67, 618-621.

Chandrasekara, A., & Shahidi, F. (2011). Determination of antioxidant activity in free and hydrolyzed fractions of millet grains and characterization of their phenolic profiles by HPLCDAD-ESI-MS^n. *Journal of Functional Food*, 3, 144-158.

Chen, Z. Y., Peng, C., Jiao, R,. Wong, Y. M., Yang, N., & Huang, Y. (2009). Anti-hypertensive nutraceuticals and functional foods. *Journal of Agricultural and Food Chemistry*, 57, 4485-4499.

Cheng, K. W., Yang, R. Y., Tsou, S. C., Lo, C. S., Ho, C. H., Lee, T. C., & Wang, M. (2009). Analysis of antioxidant activity and antioxidant constituents of Chinese toon. *Journal of Functional Food*, 1, 253-259.

Cienfuegos-Jovellanos, E., Quinones, M. M., Muguerza, B., Moulay, L., Miguel, M., & Aleixandre, A. (2009). Antihypertensive effect of a polyphenol-rich cocoa powder industrially processed to preserve the original flavonoids of the cocoa beans. *Journal of Agricultural and Food Chemistry*, 57, 6156-6162.

Fu, X. C., Wang, M. W., Li, S. P., Zhang, Y., & Wang, H. L. (2005). Vasodilatation produced by orientin and its mechanism study. *Biological and Pharmaceutical Bulletin*, 28, 37-41.

Fujita, H., Yokoyama, K., Yasumoto, R., & Yoshikawa, M. (1995). Antihypertensive effect of thermolysin digest of dried bonito in spontaneously hypertensive rat. *Clinical and Experimental Pharmacology & Physiology*, Supplement 1, 304-305.

Fujita, Y., & Yoshikawa, M. (1999). LKPNM: a prodrug-type ACE-inhibitory peptide derived from fish protein. *Immunopharmacology*, 44, 123-127.

Fusi, F., Saponara, S., Pessina, F., Gorelli, B., & Sgaragli, G. (2003). Effects of quercetin and rutin on

vascular preparations. a comparison between mechanical and electrophysiological phenomena. *European Journal of Nutrition*, 42, 10-17.

Guang, C., & Phillips, R. D. (2009). Plant food-derived angiotensin I converting enzyme inhibitory peptides. *Journal of Agricultural and Food Chemistry*, 57, 5113-5120.

Hatanaka, A., Miyahara, H., Suzuki, K., & Sato, S. (2009). Isolation and identification of antihypertensive peptides from antarctic krill tail meat hydrolysate. *Journal of Food Science*, 74, 116-120.

Horiuchi, M., Fujimura, K., Terashima, T., & Iso, T. (1982). Method for determination of angiotensin-converting enzyme activity in blood and tissue by high-performance liquid chromatography. *Journal of Chromatography*, 233, 123-130.

Inoue, K., Shirai, T., Ochiai, H., Kasao, M., Hayakawa, K., Kimura, M. & Sansawa, H. (2003). Blood-pressure-lowering effect of a novel fermented milk containing γ-aminobutyric acid (GABA) in mild hypertensives. *European Journal of Clinical Nutrition*, 57, 490-495.

Jäkälä, P., Hakala A., Turpeinen, A. M., Korpela, R., Vapaatalo, H. (2009). Casein-derived bioactive tripeptides Ile-Pro-Pro and Val-Pro-Pro attenuate the development of hypertension and improve endothelial function in salt-loaded Goto–Kakizaki rats. *Journal of Functional Foods*, 1, 366-374.

Je, J. Y., Park, P. J., Byun, H. G., Jung, W. K., & Kim, S. K. (2005). Angiotensin I converting enzyme (ACE) inhibitory peptide derived from the sauce of fermented blue mussel, *Mytilus edulis*. *Bioresource Technology*, 96, 1624-1629.

Julkunen-Tiitto, R. (1985). Phenolic constituents in the leaves of northern willows: methods for the analysis of certain phenolics. *Journal of Agricultural Food Chemistry*, 33, 213-217.

Kim, D. W., Hwang, I. K., Lim, S. S., Yoo, K. Y., Li, H., Kim, Y. S., Kwon, D. Y., Moon, W. K., Kim, D. W., & Won, M. H. (2009). Germinated buckwheat extract decreases blood pressure and nitrotyrosine immunoreactivity in aortic endothelial cells in spontaneously hypertensive rats. *Phytotherapy Research*, 23, 993-998.

Kleiber, M. (1961). *The fire of life. An introduction to animal energetics.* New York: Wiley.

Kouno, K., Hirano, S., & Kuboki, H. (2005). Effects of dried bonito (*Katsuobushi*) and captopril, an

angiotensin I-converting enzyme inhibitor, on rat isolated aorta: a possible mechanism of antihypertensive action. *Bioscience, Biotechnology, and Biochemistry*, 69, 911-915.

Koyama, M., Nakamura, C., & Nakamura, K. (2011). Changes in phenols contents from buckwheat sprouts during growth stage. *Journal of Food Science and Technology*, published online.

Krkošková, B., & Mrázová, Z. (2005). Prophylactic components of buckwheat. *Food Research International*, 38, 561-568.

Li, C. H., Matsui, T., Matsumoto, K., Yamasaki, R., & Kawasaki, T. (2002). Latent production of angiotensin I-converting enzyme inhibitors from buckwheat protein. *Journal of Peptide Science*, 8, 267-274.

Li, S., Pan, M. H., Lo, C. Y., Tan, D., Wang, Y., Shahidi, F., & Ho, C. T. (2009). Chemistry and health effects of polymethoxyflavones and hydroxylated polymethoxyflavones. *Journal of Functional Food*, 1, 2-12.

Li, S. Q., & Zhang, Q. H. (2001). Advances in the development of functional foods from buckwheat. *Critical Reviews in Food Science and Nutrition*, 41, 451-464.

Lloyd-Jones, D., Adams, R. J., Brown, T. M. et al. (2010). Heart disease and stroke statistics--2010 update: a report from the American Heart Association. *Circulation*, 121, 46-215.

Mäkinen, S., Johannson, T., Gerd, E.V., Pihlava, J. M., & Pihlanto, A. (2012). Angiotensin I-converting enzyme inhibitory and antioxidant properties of rapeseed hydrolysates. *Journal of Functional Foods*, 4, 575-583.

Matoba, N., Usui, H., Fujita, H., & Yoshikawa, M. (1999). Novel anti-hypertensive peptide derived from ovalbumin induces nitric oxide-mediated vasorelaxation in an isolated SHR mesenteric artery. *FEBS Letters*, 452, 181-184.

Matsui, T., Kudo, A., & Tokuda, S. (2010). Identification of a new natural vasorelaxant compound, (+)-osbeckic acid, from rutin-free tartary buckwheat extract. *Journal of Agricultural and Food Chemistry*, 58, 10876-10879.

Miguel, M., Contreras, M. M., Recio, I., & Aleixandre, A. (2009). ACE-inhibitory and antihypertensive properties of a bovine casein hydrolysate. *Food Chemistry*, 112, 211-214.

Miguel, M., López-Fandiño, R., Ramos, M., & Aleixandre, A. (2005). Short-term effect of egg-white hydrolysate products on the arterial blood pressure of hypertensive rats. *British Journal of Nutrition*, 94, 731-737.

Miguel, M., Manso, M. A., Martín-Alvarez, P. J., Aleixandre, A., & López-Fandiño, R. (2007). Angiotensin-converting enzyme activity in plasma and tissues of spontaneously hypertensive rats after the short- and long-term intake of hydrolysed egg white. *Molecular Nutrition & Food Research*, 51, 555-563.

Miguel, M., Recio, I., Ramos, M., Delgado, M. A., & Aleixandre, M. A. (2006). Antihypertensive effect of peptides obtained from *Enterococcus faecalis*-fermented milk in rats. *Journal of Dairy Science*, 89, 3352-3359.

Mishima, S., Yoshida, C., Akino, S., & Sakamoto, T. (2005). Antihypertensive effects of Brazilian propolis: identification of caffeoylquinic acids as constituents involved in the hypotension in spontaneously hypertensive rats. *Biological & Pharmaceutical Bulletin*, 28, 1909-1914.

Nakahara, T., Sano, A., Yamaguchi, H., Sugimoto, K., Chikata, H., Kinoshita, E., & Uchida, R. (2010). Antihypertensive effect of peptide-enriched soy sauce-like seasoning and identification of its angiotensin I-converting enzyme inhibitory substances. *Journal of Agricultural and Food Chemistry*, 58, 821-827.

Nakamura, K., Nakamura, C., Maejima, S., Maejima, M., Watanabe, M., Shiro, M., & Kayahara, H. (2005). The functionality of buckwheat sour juice. In A. M. Spanier, F. Shahidi, T. H. Parliament, C. Mussinan, C. T. Ho, & E. T. Contis, *Food Flavor and Chemistry, Explorations into the 21st Century* (pp. 350-355). Cambridge, UK: The Royal Society of Chemistry.

Nakamura, K., Naramoto, K., & Fujita, M. (2008). Blood-pressure-lowering effect of fermented buckwheat sprout and the peptides contained therein. *Peptide Science 2007*, 13-16.

Nakamura, Y., Yamamoto, N., Sakai, K., & Takano, T. (1995). Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *Journal of Dairy Science*, 8, 1253-1257.

Nakamura, Y., Masuda, O., & Takano, T. (1996). Decrease of tissue angiotensin I-converting enzyme activity upon feeding sour milk in spontaneously hypertensive rats. *Bioscience, Biotechnology, and Biochemistry*, 60, 488-489.

Okamoto, A., Hanagata, H., Matsumoto, E., Kawamura, Y., Koizumi, Y., & Yanagida, F. (1995). Angiotensin I converting enzyme inhibitory activities of various fermented foods. *Bioscience, Biotechnology, and Biochemistry*, 59, 1147-1149.

Quiñones, M., Sánchez, D., Muguerza, B., Miguel, M., & Aleixandre, A. (2011). Mechanisms for antihypertensive effect of CocoanOX, a polyphenol-rich cocoa powder, in spontaneously hypertensive rats. *Food Research International*, 44, 1203-1208.

Rafik, B., Ali, B., Didier, G., Pascal, D., Moncef, N., & Naima, N. (2012). Changes in arterial blood pressure after single oral administration of cuttlefish (*Sepia officinalis*) muscle derived peptides in spontaneously hypertensive rats. *Journal of Functional Foods*, 4, 611-617.

Russell, J. C., & Proctor, S. D. (2006). Small animal models of cardiovascular disease: tools for the study of the roles of metabolic syndrome, dyslipidemia, and atherosclerosis. *Cardiovascular Pathology*, 15, 318-330.

Sauvaget, F., Mallem, M. Y., Bucas, V., Gogny, M., Desfontis, J. C., & Noireaud, J. (2010). Positive influence of AT_1 receptor antagonism upon the impaired celiprolol-induced vasodilatation in aorta from spontaneously hypertensive rats. *European Journal of Pharmacology*, 644, 169-175.

Seki, E., Kawasaki, T., Yoshida, M., Osajima, K., Tamaya, K., Matsui, T., & Osajima, Y. (1999). Antihypertensive effect of sardine peptide and valyl-tyrosine in spontaneously hypertensive rats. *Journal of Japan Society of Nutrition and Food Sciences*, 52, 271-277.

Shin, Z. I., Yu, R., Park, S. A., Chung, D. K., Ahn, C. W., Nam, H. S., Kim, K. S., & Lee, H. J. (2001). His-His-Leu, an angiotensin I converting enzyme inhibitory peptide derived from Korean soybean paste, exerts antihypertensive activity in vivo. *Journal of Agricultural and Food Chemistry*, 49, 3004-3009.

Su, D. F., Xu, L. P., Miao, C. Y., Xie, H. H., Shen, F. M., & Jiang, Y. Y. (2004). Two useful methods for

evaluating antihypertensive drugs in conscious freely moving rats. *Acta Pharmacologica Sinica*, 25, 148-151.

Suzuki, A., Fujii, A., Yamamoto, N., Yamamoto, M., Ohminami, H., Kameyama, A., Shibuya, Y., Nishizawa, Y., Tokimitsu, I., & Saito, I. (2006). Improvement of hypertension and vascular dysfunction by hydroxyhydroquinone-free coffee in a genetic model of hypertension. *FEBS Letters*, 580, 2317-2322.

Sweet, C. S., Arbegast, P. T., Gaul, S. L., Blaine, E. H., & Gross, D. M. (1981). Relationship between angiotensin I blockade and antihypertensive properties of single doses of MK-421 and captopril in spontaneous and renal hypertensive rats. *European Journal of Pharmacology*, 76, 167-176.

Tonkin, A., & Wing, L. (1996). Management of isolated systolic hypertension. *Critical Reviews in Food Science and Nutrition*, 51, 738-749.

Ushida, Y., Matsui, T., Tanaka, M., Matsumoto, K., Hosoyama, H., Mitomi, A., Sagesaka, Y., & Kakuda, T. (2008). Endothelium-dependent vasorelaxation effect of rutin-free tartary buckwheat extract in isolated rat thoracic aorta. *The Journal of Nutritional Biochemistry*, 19, 700-707.

Vermeirssen, V., Van, C. J., & Verstraete, W. (2004). Bioavailability of angiotensin I converting enzyme inhibitory peptides. *British Journal of Nutrition*, 92, 357-366.

Watanabe, K., Fujimoto, J., Tomii, Y., Sasamoto, M., Makino, H., Kudo, Y., and Okada, S. (2009). *Lactobacillus kisonensis* sp. nov., *Lactobacillus otakiensis* sp. nov., *Lactobacillus rapi* sp. nov. and *Lactobacillus sunkii* sp. nov., heterofermentative species isolated from sunki, a traditional Japanese pickle. *International Journal of Systematic and Evolutionary Microbiology*, 59, 754-760.

Wu, C. L., Lee, C. L., & Pan, T. M. (2009). Red mold dioscorea has a greater antihypertensive effect than traditional red mold rice in spontaneously hypertensive rats. *Journal of Agricultural and Food Chemistry*, 57, 5035-5041.

Yang, Y., Marczak, E. D., Usui, H., Kawamura, Y., & Yoshikawa, M. (2004). Antihypertensive properties of spinach leaf protein digests. *Journal of Agricultural and Food Chemistry*, 52, 2223-2225.

Tables

Table 1. Contents of flavonoids and total phenols in BS and neo-FBS (mg/g DW)

	BS	neo-FBS
orientin[1]	5.8 ± 0.5	4.2 ± 0.5
isoorientin[1]	16.6 ± 0.7	12.9 ± 0.9*
vitexin[1]	11.8 ± 0.7	10.3 ± 0.3
isovitexin[1]	2.5 ± 0.4	1.7 ± 0.1
rutin[1]	34.0 ± 3.1	23.0 ± 1.2*
total phenols[2]	40.5 ± 0.3	43.0 ± 1.2

[1]As rutin equivalents. [2]As gallic acid equivalents. * $p < 0.05$, versus the BS as evaluated by a Student's *t*-test.

Figure legends

Fig. 1. Changes in SBP and DBP of 13-week-old SHRs after a single oral administration of the neo-FBS at different doses (0.010 and 0.10 mg/kg BW). The positive control was captopril (1.0 mg/kg BW) and the negative control was purified water. ○: 0.010 mg/kg neo-FBS (n = 10), □: 0.10 mg/kg neo-FBS (n = 6), ●: positive control (n = 6), ■: negative control (n = 10). Each data point and bar represents the mean ± S.E. * $p < 0.05$, ** $p < 0.01$, versus the negative control group as evaluated by Student's *t*-test. SBP, systolic blood pressure; DBP, diastolic blood pressure.

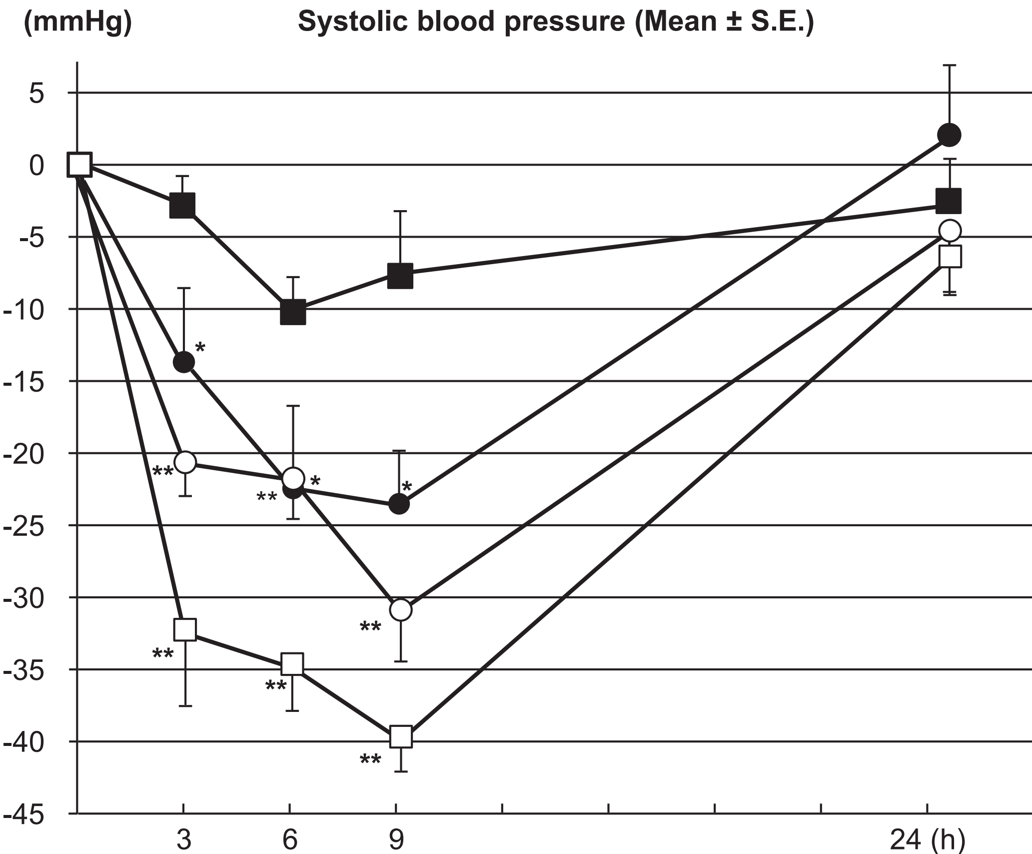
Fig. 2. ACE activity (mU/mL) in A) plasma and ACE activity (mU/mg protein) in B) the lung, C) thoracic aorta, D) heart, E) kidney, and F) liver of SHRs 6 h after administration with neo-FBS (10 mg/kg BW), captopril (positive control, 10 mg/kg BW), and purified water (negative control). Data are represented as the mean ± S.E. (n = 6). * $p < 0.05$, ** $p < 0.01$ versus the negative control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, versus the positive control group as evaluated by one-way ANOVA followed by Student's *t*-test.

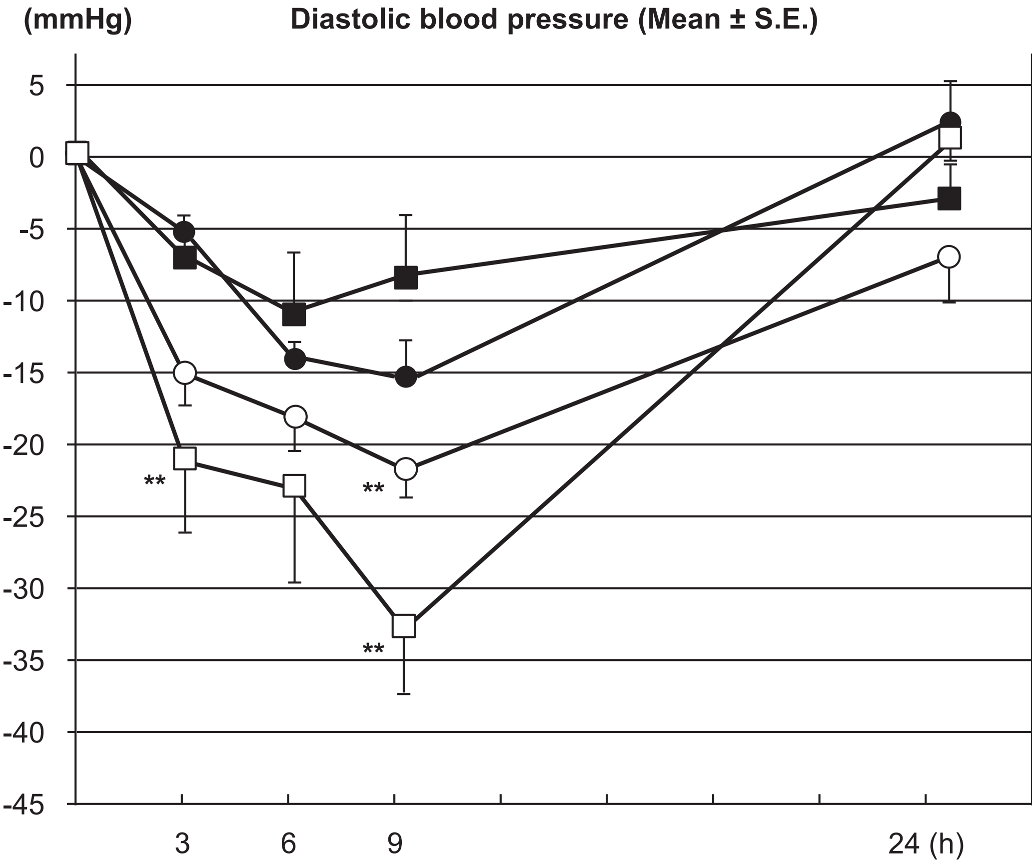
Fig. 3 Relaxation profile of thoracic aorta rings from SHRs by the neo-FBS (A) and captopril (B). The neo-FBS and captopril was added in a cumulative manner (0.50–100 μg/mL for neo-FBS, 50–1000 μg/mL for captopril) to the 0.30-μM PE-contracted aorta. PE, phenylephrine; PV, papaverine.

Fig. 4 Concentration-vasorelaxation curves of thoracic aorta rings from SHRs for neo-FBS (○) and captopril (●). Each data point and bar represent the mean ± S.E. (n = 6). * $p < 0.05$, ** $p < 0.01$, versus the tension prior to the addition of each as evaluated by Student's *t*-test.

Figure

Figure 1





Neo-FBS (0.1 mg/kg)
Neo-FBS (0.01 mg/kg)
Captopril (1.0 mg/kg)
Purified water (control)

Student *t*-test **$P < 0.01$ *$P < 0.05$

Figure
Click here to download high resolution image

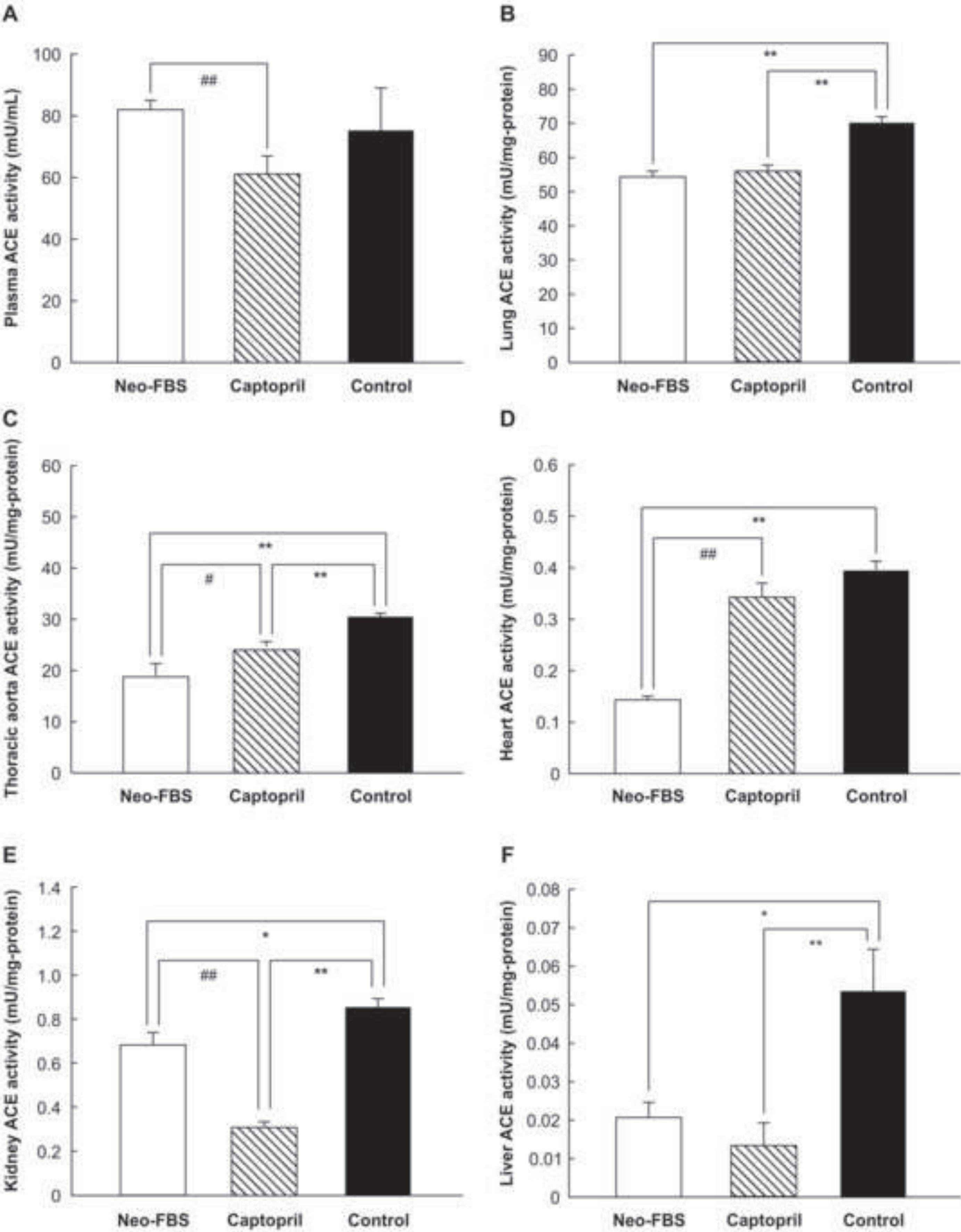
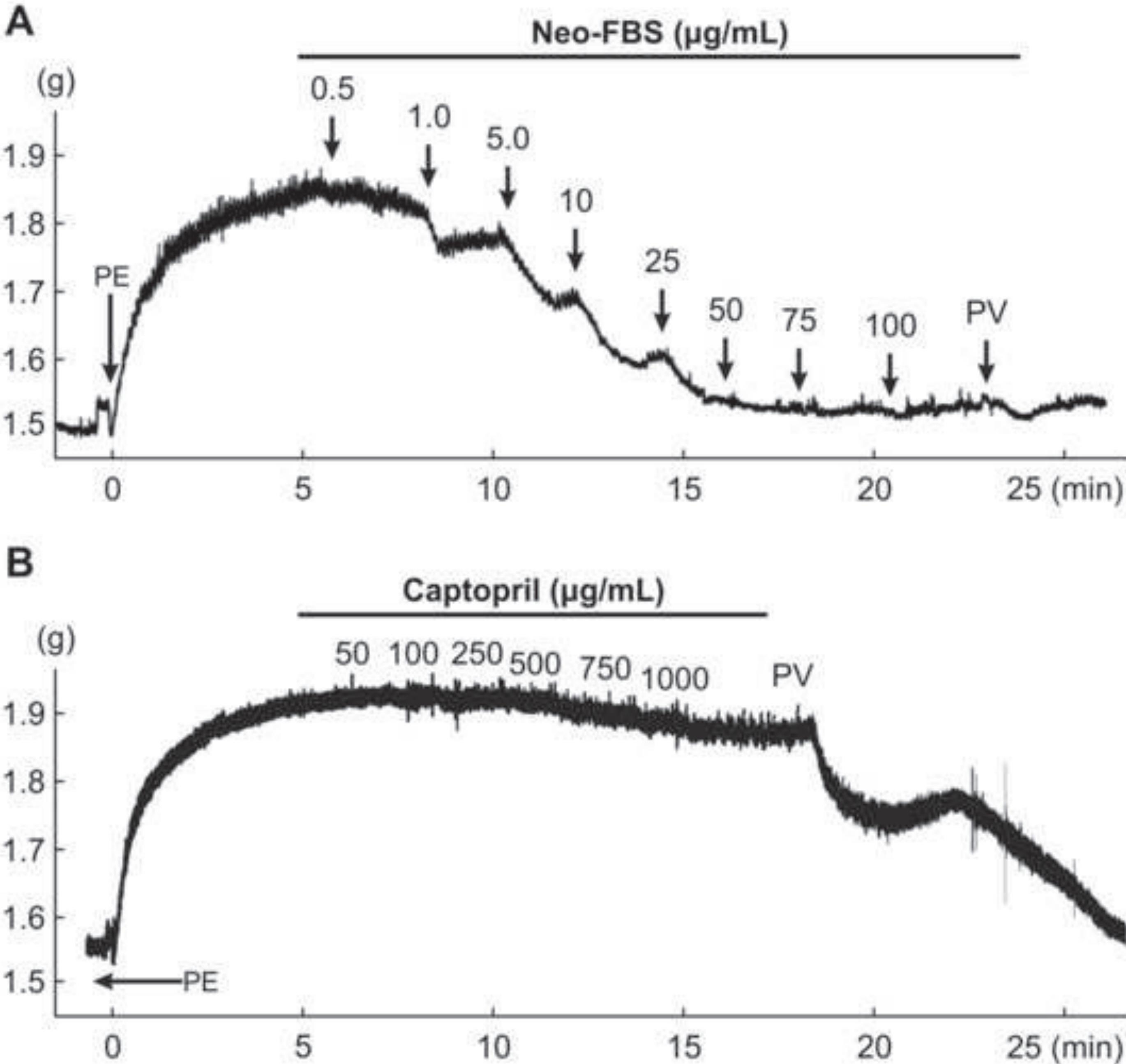


Figure
Click here to download high resolution image



Figure