

Functional Compounds in Fermented Buckwheat Sprouts

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Fermented buckwheat sprouts (FBS) are used as multifunctional foods. Their production process includes fermentation with lactic acid bacteria. The major strains were found to include *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus pentosus*, *Lactococcus lactis* subsp. *lactis*, and *Pediococcus pentosaceus* in an investigation of the lactic acid bacteria. We searched for the functional components, and nicotianamine (NA) and 2''-hydroxynicotianamine (HNA) were identified as angiotensin I-converting enzyme (ACE) inhibitors. NA and HNA increased during fermentation. Indole-3-ethanol was identified as an antioxidant (a SOD active substance), and may have been generated from tryptophan during fermentation because it was not contained in green buckwheat juice. A safety test demonstrated that FBS contained were safe functional food components, showing negative results in buckwheat allergy tests. Any buckwheat allergy substances might have been degraded during the fermentation process.

Key words: fermented buckwheat sprout; angiotensin I-converting enzyme (ACE) inhibitor; superoxide dismutase (SOD) activity; 2''-hydroxynicotianamine; indole-3-ethanol

Japanese dietary habits have markedly changed over the last 50 years, one consequence being that diseases have diversified.¹⁾ Primary prophylaxis has recently been emphasized because of the present health boom. Dietary habits are associated with disease prevention, and functional foods leading to biological regulation have attracted attention and are therefore increasingly important.

We investigated here buckwheat (*Fagopyrum esculentum* Moench) sprouts which are traditionally used as a health food. Buckwheat, which predominantly contains rutin, reduces blood pressure by inhibiting the angiotensin I-converting enzyme (ACE) and increasing blood vessel elasticity.²⁾ Buckwheat sprouts³⁾ are more nutri-

tious than their seeds. A fermented mash, obtained by fermenting buckwheat sprouts with lactic acid bacteria, is called as fermented buckwheat sprouts (FBS).

Rutin was decreased by fermentation, while quercetin was generated by the hydrolysis of rutin during fermentation. FBS showed higher ACE inhibitory activity and antioxidative activity than buckwheat sprouts; we therefore investigated the lactic acid bacteria for fermenting FBS, and biological activities such as ACE inhibition and antioxidative activity conferred by the functional compounds in FBS. Allergy and toxicity tests were also conducted to examine safety. We describe these biological activities of FBS and the identification of the active compounds.

Materials and Methods

Apparatus. Positive ion FAB-MS data were recorded with a Jeol JMS-DX 303 mass spectrometer, using *m*-nitrobenzyl alcohol as a matrix. LC/ESI-TOF-MS data were measured with a Thermo Finnigan LCQ spectrometer equipped for HPLC (Agilent Zorbax SB-18 column, 0.5 i.d. × 150 mm, 5 μm; 0.1% formic acid-MeOH solvent for gradient elution; 40 °C temp.; 210 nm detector (Agilent Technologies)). ESI-MS data were measured under the following conditions; positive ion mode, 18 °C capillary temp., sheath gas flow: 60,000 mL, 4.5 kV source voltage, 35 V capillary voltage, and 150–1,000 scan range. The ¹H-NMR (500 MHz) and ¹³C-NMR (125 MHz) spectra were measured with a Jeol Lambda 500 spectrometer in CDCl₃, using TMS as an internal standard. Preparative HPLC was performed with a Shimadzu LC-6AD instrument (C30-UG-5 column, 8.0 i.d. × 250 mm, 5 μm (Nomura Chemicals)) fitted with a Jasco MD-910 multi-wavelength detector.

Reagents. The SOD WST assay kit was purchased from Dojindo Laboratories. The angiotensin I-converting enzyme (rabbit lung), Hip-His-Leu and nicotianamine (NA) were bought from Sigma Chemicals. Anionic exchange resin and 9-fluorenylmethyloxycarbonyl were purchased from Wako Chemicals, and indole-3-ethanol was from Tokyo Chemical Industries.

FBS production. Buckwheat seeds were immersed in water. The moistened seeds were planted, sprouted, and harvested when they had

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Abbreviations: ACE, angiotensin I-converting enzyme; BLAST, basic local alignment search tool; CDCl₃, deuterated chloroform; COSY, correlation spectroscopy; dNTP, deoxyribonucleoside triphosphate; EDTA, ethylenediaminetetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; FBS, fermented buckwheat sprouts; Fmoc, 9-fluorenylmethyloxycarbonyl group; GYP, glucose yeast extract peptone; HD, hyaluronidase; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; HMBC, heteronuclear multiple-bond correlation; HNA, 2''-hydroxynicotianamine; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; MNPCE, micro-nucleated polychromatic erythrocyte; MgSO₄, magnesium sulfate; MRS, de Man Rogosa and Sharpe; NA, nicotianamine; PCR, polymerase chain reaction; SHR, spontaneously hypertensive rats; SOD, superoxide dismutase; TOF-MS, time of flight mass spectrometry

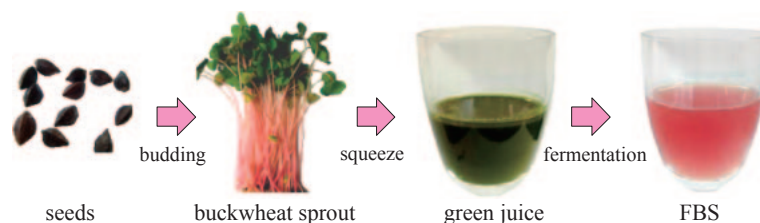


Fig. 1. Production of FBS.

grown to about 15 cm. Buckwheat green juice was subsequently obtained from the sprouts by using a juicer. Pomace was removed by filtration and then the juice was frozen. A starter culture was added during thawing to initiate fermentation, and FBS were obtained at room temperature within about 10–15 d (Fig. 1). Chlorophyll was degraded and precipitated by fermentation, resulting in the red color of FBS.

Identification of lactic acid bacteria in FBS. The lactic acid bacteria in FBS were separated and identified.⁴⁾ FBS were diluted with sterilized saline, and samples were pipetted onto the surface of MRS (Merck) or GYP CaCO₃ agar plates, each sample being spread evenly over the agar surface by using a sterile glass spreader. The plates were then incubated until the colonies appeared. Repeated selection and restreaking of a differently-colored or -shaped colony enabled pure lactic acid bacterial strains to be obtained. The 16S rRNA genes of the obtained strains were analyzed to identify the bacterial species. PCR was performed under the following conditions: initial denaturation at 94 °C for 2 min, 30 cycles with denaturation at 95 °C for 15 s, primer annealing at 58 °C for 30 s, primer extension at 68 °C for 2 min, final extension at 68 °C for 7 min, and cooling to 4 °C. The PCR reaction mixture contained 5 μ L of a 10 \times loading buffer, 5 μ L of 2 mM dNTP, 3 μ L of 25 mM MgSO₄, 1.5 μ L each of primers F (Eco F (forward primer) 10–26) and R (Eco R (reverse primer) 1541–1526), 1 μ L of KOD-Plus ver. 2 as DNA polymerase (Toyobo), 1 μ L of DNA and 33 μ L of D.W. Twenty five microliters of the PCR product was electrophoresed at 100 V for 1 h on 0.8% agarose gel (SeaKem GTG agarose: Takara Bio) in a 1 \times Tris-borate-EDTA buffer which was then stained with 2.5 μ g/mL of ethidium bromide and examined under UV light. Bands were excised and DNA was purified from the gel slices with a gel extraction kit (Qiagen). The DNA was then sequenced with an ABI Prism 310 genetic analyzer (Applied Biosystems), using the Big Dye X terminator (Applied Biosystems). Sequence data were analyzed on the Internet by using the BLAST program of the DNA Data Bank of Japan (DDBJ).

Organic acids in FBS. Organic acids were measured by the internal standard (crotonic acid) method,⁵⁾ using an LC-10A HPLC instrument (Shimadzu) equipped with a Shim-pack SCR-102H column (8.0 i.d. \times 300 mm, Shimadzu) and a CCD-6A electroconductivity detector (Shimadzu).

Measurement of the ACE inhibitory activity. The method of Kawagishi *et al.*,⁶⁾ based on that developed by Cushman,⁷⁾ was used for measuring the ACE inhibitory activity. A buffer containing 450 mL of 50 mM Na₂B₄O₇ and 550 mL of 200 mM H₃BO₃ was mixed and adjusted to pH 8.3. A substrate solution containing 7.6 mM Hip-His-Leu and 608 mM NaCl was dissolved in this buffer solution, and an ACE solution containing 0.1 unit of rabbit lung ACE was dissolved in this buffer (60 mU/mL activity). A 30- μ L amount of a sample solution and 250 μ L of the substrate solution were placed in a test tube and incubated in a thermo bath at 37 °C for 5 min. A 100- μ L amount of the ACE solution (6 mU) was then added, the mixture immediately stirred and allowed to react at 37 °C for 30 min, before 250 μ L of 1 N hydrochloric acid was added and the mixture was stirred to terminate the reaction. Ethyl acetate (1.5 mL) was finally added and the mixture stirred well, before extracting the hippurate that had been released. After centrifugating at 3,000 rpm for 10 min, 0.5 mL of the upper ethyl acetate layer was recovered and placed in a test tube. The ethyl acetate was aspirated and removed in a desiccator. After confirming the complete removal of the ethyl acetate, 4 mL of distilled water was

added and the extract was stirred well to dissolve the hippurate, before the absorbance was measured at 228 nm. The inhibitory activity was calculated as $[(E_c - E_s)/(E_c - E_b) \times 100]$, where E_s is the absorbance with the test sample added to the reaction mixture, E_c is the absorbance with water added instead of the test sample and E_b is a blank without ACE. The activity of the ACE inhibitory principle is represented by the molarity of the reaction mixture, which showed interference of 50% (IC_{50}) under these conditions.

Preliminary survey of the ACE inhibitor. Samples were prepared for the ACE inhibition test. FBS and buckwheat green juice (1,000 mL each) were separated into their supernatants and precipitates by cool centrifugation (4 °C, 8,000 rpm, 10 min). About 950 mL of each supernatant was passed through a filter (0.45 μ m pore size), concentrated by evaporation and then freeze-dried. Each freeze-dried substance was dissolved in water to be used as a sample.

Isolation of the ACE inhibitors. The freeze-dried FBS powder (1.0 g) was washed with 50 mL of 90% MeOH at 37 °C for 24 h. The resulting residue was subjected to negative ion exchange column chromatography. Anionic exchange resin (Dowex 1 \times 4, 100–200 mesh, Wako) was then added, and swelling was achieved with a 0.1 M borate buffer at pH 8.0. This was stabilized by distilled water which was then discarded. The concentration of the acetic acid was gradually increased to 0.3, 0.4, and 0.5 M, and the solution divided into fractions. Each fraction was monitored by the ninhydrin reaction and assay for ACE inhibitory activity, the 0.3 M acetic acid fraction with the highest activity being used for an identification analysis. The active fractions were chromatographed by HPLC (Agilent Zorbax SB-18 column (0.5 i.d. \times 150 mm, 5 μ m), 0.1% formic acid-MeOH solvent, gradient elution, 15 μ L/min flow rate, 210 nm detection, 40 °C column temp.). Compounds A (0.2 mg) and B (0.8 mg) with R_t 54.5 and 60.0 min showed strong ACE inhibitory activities and were therefore isolated.

Quantitative analysis of the ACE inhibitor. A sample (about 2 g) was extracted three times with EtOH-H₂O (7:3) by refluxing. The combined extract was concentrated by evaporation to a small volume and defatted by EtOAc. The aqueous layer was applied to a column (2.0 i.d. \times 100 mm) of Dowex resin (1 \times 4, AcO⁻), washed with H₂O (50 mL) and eluted with 0.5 N AcOH (100 mL). The eluate was concentrated by evaporating to dryness and dissolved in a suitable amount of the HPLC eluting solvent. The HPLC conditions were as follow: Shimadzu LC-7 instrument with fluorescence detection and post-column reaction, TSK-gel ODS-80Ts column (4.6 i.d. \times 250 mm), 0.05 M sodium laurate-acetonitrile mobile phase (6.5:3.5 v/v), 0.75 mL/min flow rate, 40 °C column temp., reaction solution of 0.2% *o*-phthalaldehyde + 0.5% 2-mercaptoethanol in a 0.2 M boric acid buffer (pH 9.9), 0.5 mL/min reaction solution flow rate, 40 °C column temp., 50 °C reaction temp., 365 nm excitation wavelength, 455 nm fluorescence wavelength.

Measurement of the SOD activity.⁸⁾ Each sample was assayed for SOD activity by using a colorimetric SOD assay kit (Dojindo Molecular Tec.). The absorbance was measured at 450 nm with a plate reader to determine the percentage SOD activity as $[(A_{\text{blank 1}} - A_{\text{blank 3}}) - (A_{\text{sample}} - A_{\text{blank 2}})] / (A_{\text{blank 1}} - A_{\text{blank 3}}) \times 100\%$.

Preliminary survey of for SOD activity. FBS (1,000 mL) was fractionated into its hexane, chloroform, ethyl acetate, butanol, and aqueous phases. Each organic phase was dehydrated and concentrated.

Isolation and identification of the SOD inhibitor. The chloroform fraction, which showed high antioxidative activity, was further separated by silica gel chromatography with SOD activity as an indicator.

Hyaluronidase (HD) inhibitory activity.⁹⁾ The HD inhibitory activity was measured by the method of Asada *et al.*¹⁰⁾ FBS, buckwheat green juice, and filter-sterilized FBS were each powdered by freeze-drying and prepared at 100 mg/mL to be tested.

Identification of keracyanine. The freeze-dried FBS powder (2.0 g) was extracted with 90% MeOH (100 mL) at 37 °C for 24 h. The obtained residue (1.6 g) was applied to ODS column chromatography. The 30% MeOH-eluted fraction (72.3 mg) was further fractionated by preparative ODS column chromatography.

Safety tests of FBS. Allergic examination tests were carried out by a FASPEK-specific kit (Morinaga Institute of Biological Science) and Fastkit ELISA (Nippon Meat Packers) to evaluate the safety of FBS. A single-dose oral toxicity test was carried out on a Crj:CD-1 mouse (Charles River). A micronucleus assay method¹¹⁾ was employed for the toxicity test and for this purpose the BDF1 mouse (Charles River) was used.

Results and Discussion

Lactic acid bacterium and organic acids in FBS

The lactic acid bacterial strains were isolated and identified to examine the microorganisms contained in FBS. Five lactic acid bacterial strains were identified in the FBS samples: *Lactobacillus plantarum* (100%), *Lactobacillus brevis* (100%), *Lactobacillus pentosus* (99%), *Lactococcus lactis* subsp. *lactis* (100%), and *Pediococcus pentosaceus* (100%). Figures in parentheses indicate the sequence similarity to the reference strains. *L. plantarum* was predominant of these strains in FBS. Of the five lactic acid bacterial strains, *L. plantarum*, *L. brevis*, *L. pentosus*, *L. lactis* subsp. *lactis*, and *P. pentosaceus* were each added as a starter strain to 500 mL of buckwheat green juice for fermentation at 25 °C. The prepared extracts were stable in the pH 3.2–3.4 range, with a favorable color and smell; these strains could therefore be used for fermentation. An analysis of the organic acids in FBS showed lactic acid, acetic acid and succinic acid to be respectively present at 109, 20.0 and 0.24 μmol/mL.

ACE inhibitors in FBS

ACE is an enzyme involved in blood pressure regulation *in vivo*. Renin acts on angiotensinogen to generate angiotensin I in the rennin-angiotensin system, and ACE acts on angiotensin I, which in turn is converted into angiotensin II to raise the blood pressure. Angiotensin II, with its potent vasopressive action, can therefore be reduced by inhibiting ACE. A test with spontaneously hypertensive rats showed the blood pressure elevation to be suppressed by FBS,¹²⁾ suggesting that an ACE inhibitor was included in FBS. The effect was confirmed *in vivo*, and the ACE inhibitory activity of FBS was therefore measured. It is well known that most ACE inhibitors are peptides. Potent ACE inhibition cannot be explained by the existing peptides, and it has been reported that buckwheat contained peptides such as Tyr-Gln-Tyr, Pro-Ser-Tyr, and Ile-Thr-Phe (IC₅₀ values of 4 μM to 23.1 μM)¹³⁾ that are ACE inhibitors. It is therefore possible that different peptides and amino acids are generated by fermentation. Sub-

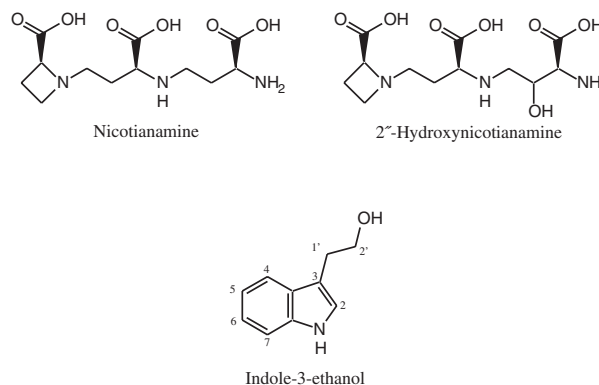


Fig. 2. Structures of Nicotianamine (NA), 2'-Hydroxynicotianamine (HNA) and Indole-3-ethanol.

stances other than peptides were also explored, peaks being detected by HPLC at *Rt* 60.0 min for NA and at *Rt* 54.5 min for HNA. FBS showed NA and HNA peaks at around 60.0 and 54.5 min.¹⁴⁾ These peaks were also detected in buckwheat green juice. HNA was identified as a 9-fluorenylmethyloxycarbonyl (Fmoc) derivative described by Wada *et al.*¹⁵⁾ HNA was therefore adjusted to a concentration of 0.8 mg/mL, Fmoc-converted, and measured by LC-MS (15.9 min retention time, ESI-MS *m/z*: 764 [M + H]⁺, 786 [M + Na]⁺) for comparison with FBS. The MS data for FBS matched those of HNA confirming that FBS contained HNA. The two amino acids, NA and HNA (Fig. 2), were therefore identified as the major ACE inhibitor in FBS and buckwheat green juice. FBS contained large amounts of NA and HNA, their contents in FBS and green juice being showed in Table 1. NA is a substance that was discovered in tobacco leaves, is a metal chelator ubiquitous among higher plants,^{16,17)} and plays an important role as an intermediate of mugineic acid in Gramineae plants. *S*-adenosylmethionine is synthesized as a trimer by NA synthase.^{18–21)} NA and HNA both have potent ACE inhibitory action, whose antihypertensive effects have recently attracted attention. NA and HNA had strong IC₅₀ action (0.085 and 0.084 μM) which was increased by fermentation. It was therefore assumed that NA and HNA were the main contributors to the ACE inhibitory activity in FBS. The ACE inhibitory activity increased by about six times when FBS was produced from the buckwheat green juice. The total amount of NA and HNA was increased by about three times by fermentation. The IC₅₀ values of NA and HNA for ACE were almost equal, and stronger than that of peptides.¹³⁾ It is therefore assumed that the contribution of NA and HNA to the total ACE inhibitory activity was approximately 50%. These results are sufficiently interesting to warrant further studies being conducted. It is thought that the ACE inhibitory activity in FBS was shown by these compounds with peptides.

SOD-active substance in FBS

Arteriosclerosis is associated with damaged vascular endothelial cells due to oxidized low-density lipoprotein (LDL). Various reactive oxygen species are generated in ischemic regions to damage the vessels. Diets with an antioxidative effect are hence effective in reducing the risks of vascular disorders. The antioxidative effect of

Table 1. ACE Inhibitory, SOD and HD Activities, and NA, HNA and Indole-3-ethanol Contents in Buckwheat Green Juice and FBS

Sample	ACE inhibitory activity (IC ₅₀) (mg/100 g)	NA* (mg/100 g)	HNA* (mg/100 g)	SOD activity (IC ₅₀) (mg/100 g)	Indole-3-ethanol (mg/100 g)	HD inhibitory activity** (%)
Buckwheat green juice	1.25	7.9	25.1	1.13	—	5.5
FBS	0.24	21.5	83.3	0.36	50	39.1

*IC₅₀ values of NA and HNA for ACE are 0.085 and 0.084 μ M.

**HD inhibitory activity (%) was determined by using water as a control and by suppressing the hyaluronic acid.

FBS was evaluated by using the superoxide dismutase (SOD) activity as a parameter.

The SOD-active compound eluted with chloroform-ethyl acetate (3:1) showed strong SOD activity. This compound had a pseudomolecular ion peak at MS m/z : 160 [M – H][–] by ESI-MS. The ¹H- and ¹³C-NMR data for this compound were obtained from 2D-NMR experiments. In ¹H-NMR (500 MHz, CDCl₃), five aromatic methins δ 7.61 (1H, d, J = 8.0, H-4), 7.36 (1H, d, J = 7.0, H-7), 7.20 (1H, dd, J = 7.0, 2.0, H-6), 7.12 (1H, dd, J = 7.0, 2.0, H-5) and 7.04 (1H, s, H-2), four methylene protons δ 3.92 (2H, t, J = 6.0, H-2') and 3.04 (2H, t, J = 6.0, H-1'), and one NH proton δ 8.14 (1H, s, NH) were observed. In ¹³C-NMR (125 MHz, CDCl₃), ten carbon signals, including eight aromatic carbons δ 136.4 (C-8), 127.4 (C-9), 122.5 (C-2), 122.2 (C-5), 119.4 (C-6), 118.7 (C-4), 112.0 (C-3) and 111.2 (C-7) and two methylene carbons δ 62.6 (C-2') and 28.5 (C-1'), were observed. HMBC and COSY experiments revealed the presence of an ethanol moiety and indole moiety. The HMBC correlations were from H-4 to C1'' and from H-2 to C1''. C1'' and C2'' revealed that the ethanol moiety was linked at C-3 to the indole moiety; the compound was therefore identified as indole-3-ethanol (Fig. 2). These spectral data are identical to previous literature values.²²⁾ The indole-3-ethanol contents in FBS and buckwheat green juice are shown in Table 1. FBS showed higher antioxidative activity than buckwheat green juice. FBS contained indole-3-ethanol, which exhibits higher antioxidative activity than ascorbic acid (vitamin C). FBS therefore showed higher antioxidative activity than buckwheat green juice. Indole-3-ethanol has been isolated from cucumber shoots and is used to generate indole-3-acetic acid to promote growth.²²⁾ Indole-3-ethanol has also been isolated from fermented vinegar extracted from soy broth, and may be a secondary metabolite of tryptophan that is generated during alcoholic fermentation by yeast.²³⁾ In addition, indole-3-ethanol is produced during tryptophan metabolism by yeast, and is generated through the functions of rice malt and yeast in alcohol.²⁴⁾ The indole-3-ethanol identified in this study might therefore have been generated from tryptophan by lactic acid bacteria through the same mechanism as that described in other literature. FBS is rich in such flavonoids as quercetin, orientin, and vitexin. These ingredients may contribute to the high antioxidative activity. The indole-3-ethanol contents in FBS and buckwheat green juice suggest that, like ACE inhibitors, a large amount of indole-3-ethanol may be generated during fermentation.²⁵⁾ It has been proposed that SOD activity was shown by indole-3-ethanol with rutin (2,300 to 600 mg/100 g of FBS) and quercetin (20 to 163 mg/

100 g of FBS).¹²⁾ It is assumed from the content of indole-3-ethanol (50 mg/100 g) that the SOD activity in FBS was mainly contributed by indole-3-ethanol.

HD inhibitors in FBS

It is estimated that 30% of Japanese people currently suffer from certain allergic diseases. We therefore examined whether FBS had an anti-allergic action. HD activity increases before histamine release in the living body. HD inhibitory activity should therefore exert an anti-allergic action because it is positively correlated with the suppression of histamine release. FBS, buckwheat green juice, and filter-sterilized FBS were each powdered by freeze-drying and prepared at 100 mg/mL to be tested. FBS showed HD inhibitory activity, while buckwheat green juice also exhibited activity, but as low as about 5% compared with FBS (Table 1). Samples from which lactic acid bacteria had been removed through a 0.45 μ m membrane filter also showed HD inhibitory activity. Thus, the FBS ingredients, but not lactic acid bacteria, may have exerted HD inhibitory activity.

Pigments in FBS

The isolated compound (0.8 mg) was identified as keracyanine from its spectral data by comparison with an authentic sample by HPLC (Develosil ODS-HG5 column, 0.1% formic acid and acetonitrile solvent, linear acetonitrile gradient (0–10% in 0–5 min, 10–17% in 10–20 min, 35–100% in 20–30 min), 35 °C column temp., 530 nm detector); R_t 17.0 min, 530 nm. Keracyanine (40 mg/100 g) was contained in FBS and found in the stem of buckwheat sprouts. It is thought that the red color of FBS from keracyanine was a result of the acidic pH value of lactic acid.

Safety of FBS

Safety tests are routinely conducted for pharmaceuticals, but not for foods; thus, any negative results are generally investigated to assure safety. An analysis of the allergenic ingredient protein level of FBS by using the FASPEK-specific kit and Fastkit showed respective values of less than 20.0 μ g/g and 25.6 μ g/g. The LD₅₀ value for single-dose oral toxicity was calculated to be over 5,000 mg/kg. The occurrence rate of MNPCE by the administering FBS was observed from a micro-nucleus assay to be 0.22% at 500 mg, 0.11% at 1,000 mg and 0.21% at 2,000 mg. All the foregoing tests showed negative results, FBS thus being proven to be safe as a food.

We carried out a study of the functional compounds in FBS and identified five strains of lactic acid bacteria.

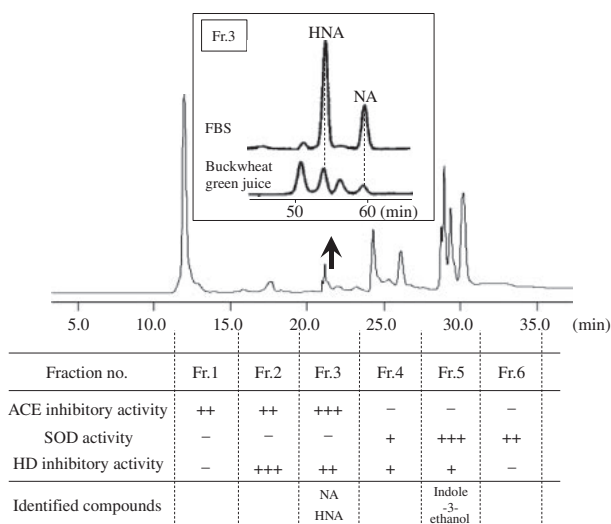


Fig. 3. Chromatograms and Activities for Each Fraction of FBS, and Expanded Chromatogram for Fr. 3.

¹Activity: +++ very strong, ++ strong, + low. ²HPLC conditions; Develosil ODS-UG-5 column (4.6 i.d. × 250 mm), water as mobile phase A, acetonitrile as mobile phase B, linear acetonitrile gradient of 0–30% in 40 min, 1.0 mL/min flow rate, 40 °C temp., 215 nm detector. ³HPLC conditions for the expanded chromatogram of fraction 3; TSK-gel ODS-80Ts column (4.6 i.d. × 250 mm), 0.05 M sodium laurate-acetonitrile (6.5:3.5 v/v) mobile phase, 0.75 mL/min flow rate, 215 nm detector, 40 °C column temp., 0.2% *o*-phthalaldehyde + 0.5% 2-mercaptoethanol compound liquid reaction solution (0.2 M boric acid buffer, pH 9.9), 0.5 mL/min reaction solution flow rate, 40 °C column temp., 50 °C reaction temp., 365 nm excitation wavelength, 445 nm fluorescence wavelength.

The results suggested two kinds of ACE inhibitory activators, NA and HNA, in FBS, and indol-3-ethanol was isolated as antioxidative compound. Table 1 and Fig. 3 show that different HPLC fractions possessed ACE, HD inhibitory and SOD activities. It is assumed that NA, HNA and indole-3-ethanol were important active substances in FBS. The results of buckwheat allergy testing with two kinds of kits were both negative, and the single dose oral toxicity test and toxicity test results all confirmed the safety of FBS. Nutritional sprout foods were fermented with lactic acid bacteria to generate functional FBS and found to be safe and useful as a functional material.

References

- 1) Nakaji S, Sugawara K, Saito D, Yoshioka Y, MacAuley D, Bradley T, Kernohan T, and Baxter D, *Eur. J. Nutr.*, **41**, 222–227 (2002).
- 2) Li C-H, Matsui T, Matsumoto K, Yamasaki R, and Kawasaki T, *J. Pept. Sci.*, **8**, 267–274 (2002).
- 3) Wijngaard HH and Arendt EK, *Cereal Chem.*, **83**, 391–401 (2006).
- 4) Kebede A, *Afr. J. Biotechnol.*, **6**, 1469–1478 (2007).
- 5) Morita T, Kasaoka S, Hase K, and Kiriyama S, *J. Nutr.*, **129**, 2081–2087 (1999).
- 6) Kawagishi S, *Japan Sci. Soc. Press*, **38**, 116–129 (1996).
- 7) Ondetti MA, Rubin B, and Cushman DW, *Science*, **196**, 441–444 (1977).
- 8) Watanabe M, *Biosci. Biotechnol. Biochem.*, **71**, 579–582 (2007).
- 9) Kakegawa H, Matsumoto H, and Satoh T, *Chem. Pharm. Bull.*, **32**, 642–646 (1985).
- 10) Asada M, Sugie M, Inoue M, Nakagomi K, Hongo S, Murata K, Irie S, Takeuchi T, Tomizuka N, and Oka S, *Biosci. Biotechnol. Biochem.*, **61**, 1030–1032 (1997).
- 11) Salamone M, Heddle J, Stuart E, and Katz M, *Mutat. Res.*, **74**, 347–356 (1980).
- 12) Nakamura K, Maejima Y, Maejima S, and Niimura E, *Pept. Sci.*, **2005**, 191–194 (2006).
- 13) Ma M-S, Bae IY, Lee HG, and Yang C-B, *Food Chem.*, **96**, 36–42 (2006).
- 14) Aoyagi Y, *Phytochem.*, **67**, 618–621 (2006).
- 15) Wada Y, Yamaguchi I, Takahashi M, Nakanishi H, Mori S, and Nishizawa NK, *Biosci. Biotechnol. Biochem.*, **71**, 435–441 (2007).
- 16) Takahashi M, Terada Y, Nakai I, Nakanishi H, Yoshimura E, Mori S, and Nishizawa NK, *Plant Cell*, **15**, 1263–1280 (2003).
- 17) Shojima S, Nishizawa NK, Fushiya S, Nozoe S, Irifune T, and Mori S, *Plant Physiol.*, **93**, 1497–1503 (1990).
- 18) Shojima S, Nishizawa NK, Fushiya S, Nozoe S, Kumashiro T, Nagata T, Ohata T, and Mori S, *Biol. Met.*, **2**, 142–145 (1989).
- 19) Kinoshita E, Yamakoshi J, and Kikuchi M, *Biosci. Biotechnol. Biochem.*, **57**, 1107–1110 (1993).
- 20) Shimizu E, Hayashi A, Takahashi R, Aoyagi Y, Murakami T, and Kimoto K, *J. Nutr. Sci. Vitaminol.*, **45**, 375–383 (1999).
- 21) Kataoka S, *J. Biosci. Bioeng.*, **100**, 227–234 (2005).
- 22) Rayle DL and Purves WK, *Plant Physiol.*, **42**, 520–524 (1967).
- 23) Inagaki S, Morimura S, Gondo K, Tang Y, Akutagawa H, and Kida K, *Biosci. Biotechnol. Biochem.*, **71**, 371–379 (2007).
- 24) Watanabe T, Yamamoto A, Nagai S, and Terabe S, *J. Chromatogr. A*, **825**, 102–106 (1998).
- 25) Cipolla-Neto J, Abdalla DSP, Markus RP, and Campa A, *J. Neural Transm.*, **92**, 117–123 (1993).