JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Optimization of Bioactive Compounds in Buckwheat Sprouts and Their Effect on Blood Cholesterol in Hamsters

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Nutrient levels in buckwheats that were maximized in day 8 sprouts (D8SP) included total phenolics, quercetin, and *L*-ascorbic acid, whereas those of oxalic, malic, tartaric, and citric acids, rutin, and γ-aminobutyric acid (GABA) were found to reach maximum levels on day 10. Ethanolic extract of D8SP (2.5 mg/mL) revealed potent free-radical scavenging (FRS) and antioxidative (ANO) capabilities. However, its Fe^{2+}-chelating capability was only moderate. To further study the hypolipidemic activity of D8SP, 36 Syrian hamsters were grouped into six groups and fed for 28 days, respectively, with (i) control meal, (ii) high fat plus high cholesterol meal, (iii) high fat plus high cholesterol plus 2.5% of buckwheat seeds, (iv) high fat plus high cholesterol plus 25% of buckwheat seeds, (v) high fat plus high cholesterol plus 2.5% of D8SP, and (vi) high fat plus high cholesterol plus 25% of D8SP. High seed meal prominently enhanced body weight gain, whereas high sprout meal exhibited the highest feed efficiency. Ratios of liver/body weight (L/B) were significantly lowered by all BS meals. Although low seed meal reduced serum total cholesterol (TC) levels ($p < 0.05$), its effect was still inferior to the high seed and sprout meals ($p < 0.01$). In contrast, serum triglyceride (TG) levels were lowered only by the high seed and sprout meals ($p < 0.05$). Alternatively, levels of serum low-density lipoprotein cholesterol (LDL-C) were significantly suppressed by all buckwheat meals ($p < 0.01$). Serum high-density lipoprotein cholesterol (HDL-C) levels were increased, however, insignificantly. Nutraceutically more meaningful is that both LDL-C/HDL-C and TC/HDL-C ratios were significantly lowered ($p < 0.01$). Apparently, hepatic TC levels were significantly reduced, whereas hepatic TG levels were totally unaffected. Conclusively, sprouting triggers a variety of nutritional changes in buckwheats. Day 8 sprouts, consisting of high polyphenolic and moderate quercetin contents, are nutraceutically maximized when hypocholesterolemic, hypotriglyceridemic, and antioxidative activities are concerned.

KEYWORDS: Buckwheat sprouts; hypocholesterolemic; hypotriglyceridemic; antioxidant; quercetin

INTRODUCTION

Polyphenolic rutin and quercetin in buckwheat had been found to increase with sprouting time (*1*). Buckwheat (*Fagopyrium esculentum* Möench) (Polygonaceae) is popularly recognized as an excellent selective antioxidant and hypolipidaemic nutrient food. It has many therapeutic uses in the treatment of hemorrhagic diseases and vessel fragility induced by hypertension (*2*). Traditionally, it is believed useful in strengthening vessel elasticity and preventing strokes and many cardiovascular diseases, as well as peptic ulcers.

The risk factors pertinently related with atherosclerosis are hypercholesterolemia, hypertriglyceridemia, and a suppressed level of *in vivo* antioxidative capability (*3*). Cholesterol feeds were reported to stimulate very low-density lipoprotein (VLDL) secretion, increase serum levels of VLDL-triglyceride (TG), cholesteryl esters (CE) and low-density lipoprotein (LDL), and conversely, suppress the high-density lipoprotein cholesterol (HDL-C) concentration. The level of HDL-C is always correlated with atherosclerosis (*4, 5*).

Sprouting may trigger the hydrolysis of triglycerides in seeds by activation of lipases and subsequently transform these nutrients into di- and tricarboxylic acid intermediates of the tricarboxylic acid (TCA) cycle. From the coupling of a series of mitochondrial oxidative phosphorylations, the energy required

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10.1021/jf072886x CCC: $40.75
Published on Web 01/24/2008

by various biochemical reactions can be generated during sprouting. Speculating that biochemically there could be a maximized level for each nutrient transformation during sprouting, we tracked the time-dependent change of unsaturated fatty acids, organic acids, total phenolics, rutin, quercetin, ∠-ascorbic acid, and γ-aminobutyric acid in sprouting buckwheats in this present study.

Preliminary *in vitro* observation indicated that maximum yields of most nutrients occurred on day 8 sprouting. To confirm its *in vivo* bioactivity, the ethanolic extract of day 8 sprouts was tested with an *in vivo* hamster model. Parameters including body weight gain, percent feed efficiency, liver/body weight ratio, serum total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), HDL-C, and the hepatic TC and TG were determined. Astonishing bioactive effects of day 8 buckwheat sprouts were thus discovered.

MATERIALS AND METHODS

Source of Buckwheats and Cultivation. Buckwheat seeds (*Fagopyrium esculentum* Möench) were purchased from the local market in Shalu County of Taichung Hsien, Taiwan. Buckwheat sprouts used for the experiment were grown in our laboratory. Briefly, buckwheat seeds (500 g), after screened out the defects, were soaked in water for 12 h. The wetted seeds were spread evenly onto 10 wooden boxes, each with a dimension of $l \times w \times h = 100 \times 50 \times 5$ cm, in which was filled with the cultivation substrate made of sand/sawwood chips = 1:3 to a height of 12 cm. A water sprayer unit was installed over each box. Watering was supplied every 4 h, each time for 5 min. The cultivation was conducted in an air-conditioned room held at a temperature ranging from 25.4 to 27.8 °C. The relative humidity was controlled at RH = 80−87%, which was recorded every day for a total period of 14 days. Sampling was performed on day 0, 4, 8, 10, and 14, respectively, during sprouting. The sprouts obtained were fresh lyophilized and stored at −20 °C for further use.

Chemicals. Cholesterol, LDL-C, HDL-C, and triglycerides were products of ICN Biomedicals, Inc. (Irvine, CA). Bioassay kits including triglycerides kit, cholesterol kit, LDL-C kit, and HDL-C kit were manufactured by Teco Diagnostics (Anaheim, CA). Acetonitrile (LC grade, purity 99%) was provided by Tomowa Chemical Co. (Japan). Authentic oxalic, citric, malic, and tartaric acids, ∠-ascorbic acid, α,α-diphenyl-β-picrylhydrazyl (DPPH) free radicals, linoleic acid, butylated hydroxyanisole (BHA), rutin, quercetin, γ-aminobutyric acid (GABA), and decanoic acid were products of Sigma Chemical Co. (St. Louis, MO). Waters AccQ·Fluor reagent kit was provided by Waters Associate Co. (Denver, CO). Ethylenediaminetetraacetic acid (EDTA) was purchased from Mallinckrodt Co. (Hazelwood, MO). Phosphotungstic acid was a product of Baker Analytical Reagent (Phillipsburg, NJ).

Analysis for Fatty Acids. Following the method of the American Oil Chemists' Society (AOCS), an amount of crude fat (0.1 g) extracted from either buckwheat seeds or sprouts was accurately weighed and transferred into a 50 mL round-bottom flask (*6*). Saponification and isolation of fatty acids were conducted by following the method as instructed. The combined organic solvent extracts were dehydrated with anhydrous sodium sulfate. The final dehydrated organic layer was filtered through a 0.45 μm micropore and analyzed with gas chromatography (GC). For fatty acids analysis, an flame ionization detector (FID)-type GC (Angilent 6890, Wilmington, DE) was installed with a DB-wax capillary (l = 60 m; i.d. = 0.25 mm; film thickness = 0.25 μm). Nitrogen gas was used as the carrier gas, operating at a flow rate of 1 mL/min. The column temperature was initially operated at 240 °C, programmed with an elevation rate of 2 °C/min up to 280 °C, and maintained for 10 min. An internal standard decanoic acid (0.0566 g) was used as a fatty acid marker.

Determination of Organic Acids. Buckwheat seeds or dried sprouts (600 mg) were crushed immediately before extraction, to which 50 mL of ethanol (80%) was added to macerate with agitation to facilitate the extraction. The extraction was repeated 3 times. The combined extracts were filtered through a 0.45 μm micropore (13 mm Millex filter millipore). The filtrate was evaporated at 50 °C under vacuum to dryness (This dried buckwheat ethanolic extract will be named hereafter as DBEE in the text). To redissolve DBEE for subsequent treatments, the remaining procedures were performed according to Ajlouni et al. (*7*). An aliquot of 20 μL of the filtrate was injected for high-performance liquid chromatography (HPLC) analysis (HPLC-type Hitachi L-2130, Hitachi, Japan). Calibration curves were established following the same procedures using respective authentic organic acid. The column RP-18GP250 Mightysil (l = 250 mm; i.d. = 4.6 mm; membrane thickness = 2.5 μM; Kanto Chem. Co., Inc., Japan) was used for HPLC analysis. The mobile phase used was 0.2 M KH_2PO_4, previously adjusted to pH 1.5 with H_3PO_4. For quantification, absorbance was measured at 214 nm with an UV detector (L-2400 UV detector, Hitachi, Japan). The flow rates of the eluent were controlled constantly at 1.0 mL/min during the entire analytical course.

Determination of Total Polyphenolics. Authentic gallic acid and desired amounts of DBEE were respectively dissolved in a methanol/water (60:40, v/v) mixed solvent previously acidified with HCl (0.3%) to obtain desired concentrations as indicated. An aliquot (0.2 mL of each) was added with Na_2CO_3 solution (0.8 mL, 7.5%). After the solution was well-mixed, 1 mL of Folin−Ciocalteau phenol reagent was added. The mixture was left to stand for 30 min at ambient temperature. The absorbance was measured at 765 nm. A calibration curve was established using a different concentration of authentic gallic acid treated with the same procedures, from which the phenolic contents were calculated (*8*). The amount of polyphenolics was expressed as gallic acid equivalent (GAE) in mg/100 g of extract on a dry basis.

Determination of Rutin and Quercetin. The method described by Kohara et al. (*9*) was modified. In brief, 1 g of pulverized buckwheat (seeds or dried sprouts) was accurately weighed, to which ethanol (20 mL) was added. The mixture was heated at 35 °C in a water bath with continuous agitation for 24 h. The extract was cooled to ambient temperature and added with ethanol to make a volume of 20 mL. The solution was filtered with a 0.45 μm micropore (13 mm Millex filter millipore). Aliquots of 20 μL of the filtrate were analyzed with HPLC. Similarly, the calibration curves for rutin and quercetin were established by dissolving, respectively, each desired authentic sample in methanol. The sample and standard solutions were, respectively, filtered with a 0.45 μm micropore. Aliquots (20 μL) of each filtrate were injected into the injection port and analyzed with HPLC (Hitachi L-2130 pump, Hitachi, Japan). The column used was RP-18GP250 Mightysil (l = 250 mm; i.d. = 4.6 mm; thickness = 0.32 μm) (Kanto Chemical Co., Inc., Japan). The mobile phase was programmed using solution A (phosphoric acid 85% solution/water = 99.7:0.3, v/v), solution B (acetonitrile), and solution C (methanol). From the initial zero time to the first 10 min, only solution A was used as the eluent. From 10 to 30 min, elution was conducted with A/B = 85:15. From 30 to 40 min, elution was conducted with a solvent consisting of A/B/C = 70:20:10; from 40 to 55 min, elution was conducted with A/B/C = 10:75:15; from 55 to 56 min, elution was conducted with A/B/C = 5:80:15; and from 56 min on, only solution A was used. The flow rates were controlled constantly at 1.0 mL/min through the entire analytical procedures. The detector used was L-2400 UV-type (Hitachi, Japan). The absorbance was measured at 350 nm. Calibration curves were established by plotting the peak area exhibited versus each corresponding concentration used, from which the content of each constituent in buckwheats was calculated.

Determination of ∠-Ascorbic Acid. ∠-Ascorbic acid was determined according to AOAC using the chlorophenolindophenol method (*10*). To 10 g of dried buckwheat sample, 50 mL of metaphosphoric acid solution (5%) was added. The following procedures were performed as instructed.

Determination of γ-Aminobutyric Acid. The method of Yoshhara and Suriwara was followed (*11*). To 10 g of buckwheat sample, either seeds or dried sprouts, 100 mL of ethanol (75%) was added. The mixture was heated at 85 °C for 2 min. The extraction was repeated 3 times. The combined extracts were filtered through a 0.45 μm micropore (13 mm Millex filter millipore). The filtrate was evaporated at 45 °C on a water bath. The residue was dissolved in HCl (0.6 N) and made to a volume of 2 mL. The HCl solution was filtered through a 0.45 μm micropore (13 mm Millex filter millipore). The filtrate was subjected to an amino acid derivation kit to obtain derivatives as directed by the

manufacturer. An aliquot of 10 μL was measured, injected into the injection port, and analyzed with HPLC. Authentic GABA was dissolved in HCl (0.6 N) and filtered with a 0.45 μm micropore (13 mm Millex filter millipore). Similar procedures were followed to establish a calibration curve with an authentic sample by plotting the peak area exhibited versus the corresponding concentration used, from which the content of each constituent in buckwheats was calculated. The HPLC used was Hitachi L-2130 pump (Hitachi, Japan). The column used was Nova-PakC18 column (l = 150 mm; i.d. = 3.9 mm, thickness = 4 μm). The mobile phase used was programmed by combined use of solvent AccQ Tag eluent (solvent A), acetonitrile (solvent B), and deionized water (solvent C). From zero time to the first 0.5 min, only solvent A performed elution. From 0.5 to 18 min, elution was conducted with A/B/C = 99:1:0; from 18 to 19 min, elution was conducted with A/B/C = 95:5:0; from 19 to 28 min, elution was conducted with A/B/C = 91:9:0; from 28 to 35 min, elution was conducted with A/B/C = 83:17:0; and finally, from 35 to 38 min, elution was conducted with A/B/C = 0:60:40. From 38 min on, elution was again carried out with only A. The elution flow rates were controlled at 1.0 mL/min throughout the entire experiment. The detector used was the type L-2480 fluorescence detector (Hitachi, Japan). The excitation and emission wavelengths were 250 and 390 nm, respectively.

Determination of DPPH Free-Radical Scavenging Capability. Following the method described by Shimada et al. (*12*), the DBEE was redissolved in ethanol (75%) to a concentration of 20 mg/mL to serve as a stock. For analysis, the stock solution was diluted with ethanol to 0.5−20 mg/mL [the buckwheat ethanol extract (BEE)]. A total of 4 mL of BEE (0.5–20 mg/mL) was mixed with 1 mL of freshly prepared methanolic 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. The mixture was left to stand in the dark for 30 min. The optical density was measured at 517 nm with a Hitachi U-2001 spectrophotometer. α-Tocopherol and BHA were used as positive controls. The percent free-radical scavenging capability (FRSC) was calculated by eq 1

$$\%\ \mathrm{FRSC} = [(A_{\mathrm{c}\,517\,\mathrm{nm}}) - (A_{\mathrm{s}\,517\,\mathrm{nm}})(A_{\mathrm{c}\,517\,\mathrm{nm}})] \times 100 \qquad (1)$$

where $A_{\mathrm{c\ 517\ nm}}$ is the optical density of the control at 517 nm and $A_{\mathrm{s\ 517\ nm}}$ is the optical density of the sample at 517 nm.

Determination of Ferrous Ion Chelating Capability. According to Dinis et al., 1 mL of BEE (0.5–20 mg/mL) was added with methanol (3.7 mL) and ferric chloride solution (2 mM, 0.1 mL). The mixture was left to stand for 30 s to facilitate the reaction. Ferrozine (5 mM, 0.2 mL) was added. The mixture kept on proceeding the reaction for 10 min. When the reaction was completed, the optical density was immediately taken at 562 nm with a spectrophotometer (Hitachi U-2001). EDTA was used as the positive control. The percent ferrous ion chelating capability (FICC) was calculated by eq 2

$$\%\ \mathrm{FICC} = [(A_{\mathrm{c}\,562\,\mathrm{nm}}) - (A_{\mathrm{s}\,562\,\mathrm{nm}})(A_{\mathrm{c}\,562\,\mathrm{nm}})] \times 100 \qquad (2)$$

where $A_{\mathrm{c\ 562\ nm}}$ is the optical density of the control at 562 nm and $A_{\mathrm{s\ 562\ nm}}$ is the optical density of the sample at 562 nm.

Determination of the Anti-oxidative Capability on Lecithin Lipid Micelles. To lecithin (300 mg), 30 mL of $Na_2HPO_2-NaH_2PO_2$ buffer (pH 7.4, 20 mM) [metaphosphoric acid buffer (MPB)] were added. The mixture was sonicated at 20,000 Hz for 10 min at ambient temperature. A homogeneous suspension containing 10 mg/mL lipid micelles was obtained. A reaction mixture was prepared by mixing 2 mL of the lipid micelle suspension, 0.1 mL of $FeCl_3$ solution (25 mM), 0.1 mL of ⁄-ascorbic acid (25 mM), and 1.2 mL of MPB. To the reaction mixture, 0.5 mL of BEE solutions (0.5–20 mg/mL) was added to obtain, respectively, the desired concentrations. BHT (20 mg/mL, 1 mL), TBA (1%, 2 mL), and TCA (2.8%, 1 mL) were sequentially added. A blank was performed in parallel using a same amount of MPB instead of BEE (*14*). The mixture was incubated initially at 37 °C for 2 h and then heated at 100 °C in a water bath for 20 min. After the mixture was cooled to ambient temperature, the optical density was measured at 532 nm. α-Tocopherol and BHA were used as the positive controls.

Animals and Diets. A total of 36 Syrian hamsters, aged 6–7 weeks, were purchased from the National Laboratory Animal Centre. For the first 2 weeks, the hamsters were acclimated by supplying only common market feeds. Then, the hamsters were randomly grouped by body weight into six groups (two hamsters in each stainless cage) and fed on different meals: the control group C fed on regular meals; the high fat plus high cholesterol meal (group H); the high fat plus high cholesterol plus buckwheat seeds (2.5%) (group BS-1); the high fat plus high cholesterol plus buckwheat seeds (25%) (group BS-2); the high fat plus high cholesterol plus buckwheat sprouts (2.5%) (group BSP-1); and finally, the high fat plus high cholesterol plus buckwheat sprouts (25%) (group BSP-2). On the basis of the formulation described in AIN-76 (*15*), the meals were prepared according to the formula listed in **Table 1**. Corn oils were dispensed up to 15% with 0.5% of cholesterol added for each group, except the control. Each meal was thoroughly mixed to ensure a homogeneous compositional distribution. The animal room was conditioned at 24 ± 1 °C with a relative humidity maintained at 40–60%. The light cycle was changed every 12 h. Water and meal takings were ad libidum. Body weight and amount of meals uptaken were recorded every 2 days until the end of the experiment. After 4 weeks of feeding, the hamsters were fasted for 12 h before being sacrificed. The blood and liver were collected for further analysis.

Table 1. Ingredients of the Experimental Animal Meals (%)[a]

	group					
ingredients	C	H	BS1	BS2	BSP-1	BSP-2
casein	20.0	20.0	19.8	17.7	19.6	16.1
sucrose	6	6	6	6	6	6
corn starch	58.8	45.8	46.0	26.1	46.3	30.0
corn oil	5.0	15.0	15.0	14.5	14.9	14.2
cholesterol		0.5	0.5	0.5	0.5	0.5
minerals	4	4	4	4	4	4
vitamins	1	1	1	1	1	1
choline	0.2	0.2	0.2	0.2	0.2	0.2
cellulose	5	5	5	5	5	5
seeds			2.5	25		
sprouts					2.5	25
total	100	100	100	100	100	100

[a] On the basis of AIN-76 formula [American Institute of Nutrition (AIN)]. Amounts of corn oil, buckwheat seeds, and sprouts added were based on percent weight.

Determination of Serum Lipoproteins. Experimental hamsters were first fasted for 12 h and then ether anesthesized. Blood was collected from the abdomen artery. The whole blood obtained was left to stand for 10 min and then centrifuged at 3200 rpm (KUBOTA-3740) for 20 min. Serum was collected for further assay of triglycerides, total cholesterol, LDL-C, and HDL-C (*16*).

The enzymatic CHOD-PAP method was used for determination of serum total cholesterol using a TECO DIAGNOSTICS kit. Briefly, to 10 μL of sample serum obtained, a reaction mixture (1.0 mL) was added as directed by the manufacturer. The mixture was thoroughly mixed and incubated at 37 °C for 10 min to facilitate the reaction. The absorbance was measured at 520 nm. A reference cholesterol control (10 μL) supplied by manufacturer was treated with the same procedure for calibration. Alternatively, the enzyme GDP-PAP triglyceride kit (TECO DIAGNOSTICS) was used to determine the serum triglyceride content by following the instructions given by the manufacturer. Briefly, sample serum obtained (10 μL) was measured, added with 1 mL of reaction solution, and mixed well. The reaction mixture was incubated at 37 °C for 5 min. The absorbance was taken at 520 nm. A reference triglyceride control (10 μL) supplied by the manufacturer was treated with the same procedure for calibration. For determination of LDL-C, an accurately measured amount of serum sample (5 μL) was added with LDL-C reagent 1 (0.3 mL), mixed thoroughly, and incubated at 37 °C for 5 min. After centrifugation at 3200 rpm for 20 min, the supernatant was separated and added with 0.1 mL of LDL-C reagent 2. The mixture was incubated at 37 °C for another 5 min. The amount of final colored product produced was measured spectrophotometrically at 600 nm. A reference LDL control (5 μL) supplied by manufacturer was treated with the same procedure for calibration. For determination of HDL-C, serum (5 μL) was measured and added with 0.3 mL of HDL-C precipitating reagent 1 (phosphotungstic and magnesium chloride). The reaction mixture was centrifuged at 3200 rpm for 20

Table 2. Sprouting-Time-Dependent Concentration Changes of Some Bioactive Nutrients Suspected To Be Relevantly Related to Hypolipidaemic Activities[a]

	time sprouting (day)				
content (mg/100 g)	0	4	8	10	14
palmitic acid	10 ± 0^{A}	10 ± 2^{A}	10 ± 2^{A}	10 ± 1^{A}	10 ± 1^{A}
oleic acid	30 ± 2^{A}	30 ± 4^{A}	20 ± 2^{B}	20 ± 3^{C}	20 ± 2^{D}
linoleic acid	30 ± 1^{A}	30 ± 1^{A}	30 ± 1^{B}	30 ± 5^{B}	30 ± 4^{B}
linoleinic acid	2.0 ± 0^{A}	2.0 ± 1^{A}	3.0 ± 0^{B}	3.0 ± 0^{C}	3.0 ± 0^{C}
oxalic acid	10 ± 1^{A}	80 ± 9^{B}	130 ± 13^{C}	240 ± 10^{D}	40 ± 8^{E}
malic acid	130 ± 3^{A}	1370 ± 66^{B}	2680 ± 37^{C}	6390 ± 114^{D}	1580 ± 109^{B}
tartaric acid	200 ± 13^{A}	1320 ± 26^{B}	1990 ± 57^{C}	2850 ± 14^{D}	1780 ± 87^{C}
citric acid	40 ± 4^{A}	1400 ± 217^{B}	2490 ± 145^{C}	3300 ± 71^{D}	850 ± 10^{B}
total phenolics	176.1 ± 1.1^{A}	235.4 ± 1.0^{B}	693.8 ± 1.7^{C}	629.8 ± 1.9^{C}	163.9 ± 1.6^{A}
rutin	3.5 ± 0.2^{A}	28.3 ± 0.2^{B}	174.3 ± 3.6^{C}	238.2 ± 2.6^{D}	78.4 ± 0.4^{E}
quercetin	0.4 ± 0.0^{A}	0.6 ± 0.0^{B}	4.4 ± 0.1^{C}	3.6 ± 0.1^{D}	1.3 ± 0.0^{E}
ℒ-ascorbic acid	10.0 ± 0.7^{A}	21.7 ± 0.4^{B}	26.5 ± 0.1^{C}	25.3 ± 0.5^{C}	24.1 ± 0.2^{C}
γ-aminobutyric acid	2.0 ± 0.3^{A}	11.6 ± 2.8^{B}	74.8 ± 1.7^{C}	77.0 ± 2.8^{C}	3.2 ± 1.0^{D}
ethanolic extract (%)	3.49 ± 0.1^{A}	3.41 ± 0.1^{A}	3.43 ± 0.4^{A}	3.47 ± 0.2^{A}	3.45 ± 0.2^{A}

[a] Three individual experiments were performed. Values are expressed as mean ± standard deviation (SD) ($n = 6$). Values in each row with different letters are significantly different ($p < 0.05$) from each other when compared by Duncan's multiple range tests.

min. The supernatant was separated and added with HDL-C reagent 2 (0.1 mL). The mixture was incubated at 37 °C for another 5 min to facilitate the enzymatic reaction. Finally, the optical density was read at 600 nm. A reference HDL-C control (5 μL) supplied by the manufacturer was treated with the same procedure for calibration. For analysis of hepatic lipids, Syrian hamsters were sacrificed upon completion of the feeding experiment. Livers were excised. After the livers were rinsed with Ringer solution, they were wiped gently with tissue paper to get rid of any moisture adsorbed on the surface before their weights were measured. Preparation of hepatic lipid extracts was performed according to Folch et al. (*17*). The final chloroform/methanol (2:1) extracts were combined and made to a volume of 10 mL with the same extraction solvent and stored at −20 °C for further analysis. Hepatic lipid extracts (10 μL) was transferred into a 1.5 mL centrifuge tube. Similar procedures were performed as mentioned above.

Statistics. Data obtained in the same group were analyzed by Student's *t* test with computer statistical software SPSS 10.0 (SPSS, Chicago, IL). Statistical analysis system software was used to analyze the variances, and Duncan's multiple range tests were used to test their significances of difference between paired means. Significance of difference was judged by a confidence level of $p < 0.05$.

RESULTS AND DISCUSSION

Change in Fatty Acid Composition during Sprouting. During sprouting, levels of palmitic and linoleic acids remained unchanged, while contents of oleic acid and linoleinic acid inversely changed during sprouting (**Table 2**). Such a result is virtually consistent with that previously reported (*18*). Moreover, although well-recognized that n-6 polyunsaturated fatty acids (n-6 PUFA) are very beneficial to human health, their low contents in buckwheat sprouts (**Table 2**) encouraged us to eliminate such a possibility.

Change in Organic Acid Levels during Sprouting. HPLC analysis assigned the retention times of 2.55, 3.53, 2.86, and 5.64 min to oxalic, malic, tartaric, and citric acids, respectively (figure not shown). Levels of these organic acids all similarly increased to maximum values on day 10 and then decreased drastically on day 14 during sprouting (**Table 2**). The increments were 2.4-, 49-, 14.3-, and 83-fold, respectively. As well-known, citric, oxalic, and malic acids play important roles in the citric acid cycle (the TCA cycle); these increments are definite evidence that sprouting had activated the TCA cycle. Activation of the TCA cycle is pertinently indispensable to generate the energy supply, such as ATP, in the very first step of sprouting. These carboxylic and hydroxyl groups bearing organic acids (**Table 2**) are capable of chelating many heavy metallic ions (such as Cu^{2+} and Fe^{2+}), leading to effective suppression on the oxidation of ℒ-ascorbic acid and lipid peroxidation (*19*). *In vitro*, organic acids can chelate the ferrous ion to form complexes. From such a mechanism, the *in vitro* oxidation of ℒ-ascorbic acid and lipids can be prevented. Generally, these chelated complexes can be readily absorbed because of increased solubility when complexed. However, the organic moiety of which could be readily degraded with the chelated metallic ions re-released immediately once it was consumed. Thus, the deficiency problem of such essential metallic ions could not be observed.

Change in Phenolic Contents during Sprouting. Buckwheat seeds were found to contain unusually high total polyphenolic content (176.1 mg/100 g) (**Table 2**), which increased rapidly to a maximum of 3.94-fold ($p < 0.05$) in day 8 sprouts (**Table 2**). Previously, Christeel et al. reported buckwheat powder and hulls had much higher contents (*20*).

As demonstrated, the retention times of rutin and quercetin in HPLC were 22.51 and 38.61 min, respectively. Quercetin, hyperin, rutin, protocatechuic acid, and 3, 4-dihydroxybenzaldehyde are the major antioxidants in buckwheat hulls (*21*). Holasova et al. (*22*) found higher rutin content contained in buckwheat seeds (184 mg/kg), exceedingly higher than that found in this paper, barley, and oats (**Table 2**).

Similar to total phenolics, the amount for rutin increased by 68-fold (**Table 2**). However, the rutin concentration (**Table 2**) was merely very close to that in hulls (*21*). Rutin contents in buckwheat reported elsewhere ranged from 12.6 to 35.9 mg/100 g of dry weight, which are comparable to that of day 4 sprouts but far less than that of day 10 sprouts (**Table 2**), evidencing that sprouting induced a higher content of rutin transformed from other yet unknown nutrients. In contrast, Danila et al. reported that Japanese buckwheat flour contains rutin (12.7 mg/100 g), catechin (3.3 mg/100 g), epicatechin (20.5 mg/100 g), and epicatechin gallate (1.27 mg/100 g) (*23*). Quercetin is one of the most important isoflavonoids that has been widely investigated. Its content reached a maximum on day 8 by 11-fold (**Table 2**). Virtually, quercetin content in hulls is 6.3-fold higher than in seeds (*21*).

Change of ℒ-Ascorbic Acid Levels. The concentration of ℒ-ascorbic acid varied from 10.0 mg/100 g to a maximum value of 26.5 mg/100 g on day 8. As well-known, by the method of chlrophenol indolphenol titration, the dehydroascorbic acid form could not be detected and, hence, could possibly result in a great deviation accounting for the total ascorbic acid. Although Wen et al. pointed out ℒ-ascorbic acid to be capable of reducing LDL

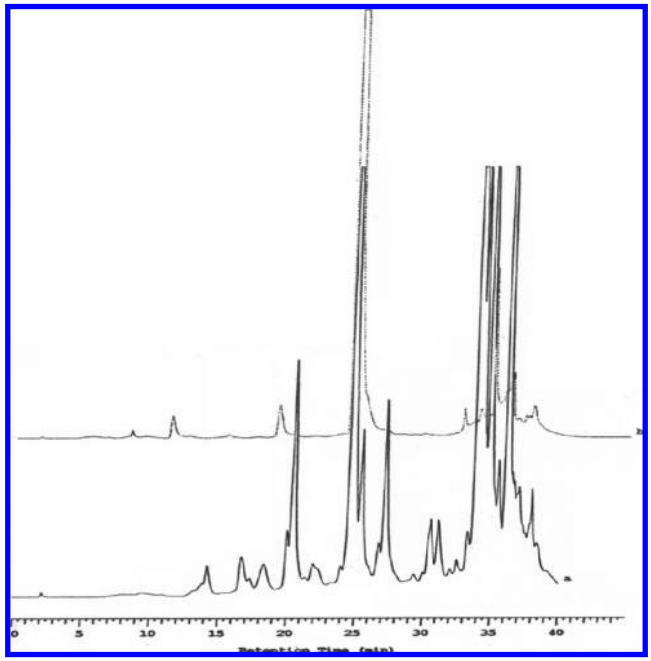

Figure 1. HPLC analysis for γ-aminobutyric acid (Top, standard GABA, $t_R = 24.73$ min; bottom, GABA extracted from day 8 buckwheat sprouts, $t_R = 24.65$ min). Buckwheat seeds or dried day 8 sprouts were extracted with ethanol. The extracts were filtered, evaporated, and redissolved in 0.6 N HCl. Authentic GABA was dissolved in HCl (0.6 N). Both extracts and authentic solutions were filtered through 0.45 μm micropore. The mobile phase used was programmed by changing proportions of solvent AccQ Tag eluent (solvent A), acetonitrile (solvent B), and deionized water (solvent C), as described in the text at a constant flow rate of 1.0 mL/min through the entire experiment. Quantification was determined by a type L-2480 fluorescence detector (Hitachi, Japan) with an excitation wavelength at 250 nm and emission wavelength at 390 nm.

oxidation, scavenging free radicals, delaying the consumption rates of intracellular membrane levels of α-tocopherol and glutathione (*24*), its effect was negligible in this regard.

Change of GABA Levels. HPLC analysis indicated a retention time of 24.73 min for the authentic GABA and 24.65 min for GABA extracted from day 8 sprouts (**Figure 1**), reaching an increase of 38.5-fold by maximum contents on day 10 (**Table 2**). As well-known, GABA is a transmitter for the central nervous system (CNS). GABA exhibits hypolipidaemic and hypocholesterolemic effects (*25*). GABA accelerates metabolism in the brain, enhances memory, and prevents atherosclerosis (*11*). Up to the present, GABA has not been well-studied with respect to its hypolipidaemic and hypoglycemic effects (*1*). GABA could exert a potential stimulating effect on β cells of the pancreas, resulting in enhanced insulin secretion that is beneficial to the treatment of diabetes mellitus (*26*). Such an effect may partly contribute to its hypolipidaemic effect.

Change of Free-Radical Scavenging Capability during Sprouting. Because total phenolics, rutin, quercetin, and ∠-ascorbic acid are closely related to antioxidative activity, to understand whether day 8 buckwheat sprouts could also concomitantly exhibit maximum free-radical scavenging and antioxidative activities, we further performed tests on its ethanolic extracts obtained from different sprouting times with their related FRSC compared. Although the yields of different BEEs were comparable ($p > 0.05$) (**Table 2**), their FRSCs greatly varied. Day 8 BEE (2.5 mg/mL) revealed a potent free-radical scavenging and antioxidant capability of 87%, approaching those of BHA and α-tocopherol. At 5 mg/mL, day 0 extract revealed only a FRSC of 49.5% (in seeds), which was increased to a maximum of 89.3% on day 8 during sprouting. The FRSC of day 8 BEE was approximately very close to that of α-tocopherol (90.6%) (**Figure 2**).

Change of Ferrous Ion Chelating Capability during Sprouting. Normally, lipid peroxidation can initiate the formation of secondary free radicals, which are damaging to human tissues (*27*). Among all heavy metallic ions, Fe^{2+} ions have been considered to be the most effective and potent oxidation acceleration entity. The ethanolic extract of buckwheat sprout of day 0 (i.e., from seeds) at 0.5 mg/mL revealed a FICC power of 11.2%; only a slight increase was found for day 4 BEE; and no apparent dose-dependent tendency was observed (**Figure 3**). At a concentration of 2.5 mg/mL, it demonstrated only 35% of moderate Fe^{2+}-chelating capability. To reach a chelating power of 63.2%, an unusually high dose of 20 mg/mL of day 8 or 10 BEE was required (**Figure 3**), which practically was too high of a dosage for use. To link the chelating mechanism, the chelating power could be attributed to high citric acid content (**Table 2**).

Change of Antioxidative Capability during Sprouting. Surprisingly, the BEE from buckwheat seeds at a concentration below 0.5 mg/mL was shown to be totally lacking antioxidant activity (**Figure 4**). Sprouting increased antioxidative capability (ANOC) of BEE; 38.8% of ANOC was reached by 0.5 mg/mL of day 8 BEE, although it was still far inferior to α-tocopherol and BHA. At a dose of 2.5 mg/mL, day 8 BEE revealed 86% of ANOC. Although effects of day 4, 8, and 10 BEE were seen very comparable at a dose of 5 mg/mL, the economical condition apparently would favor the use of day 8 BEE at 2.5 mg/mL (**Figure 4**). Wen et al. indicated that ∠-ascorbic acid was capable of suppressing LDL oxidation, scavenging free radicals, and reducing or delaying the consumption rates of intracellular ∠-ascorbic acid, α-tocopherol, and glutathione (*24*). Overall, a tendency to decrease from day 14 on (**Table 2**) might be an indication of the exhaustion of nutrients that originally had been presented in seeds that were already transformed into other biochemicals.

Change in Organ Weights. Upon feeding with BS and BSP, the body weight of the hamsters increased apparently with the daily weight gain during a feeding period of 28 days. Group BS2 exhibited the highest average body weight of 116 g ($p < 0.05$) (**Table 3**), a consequence speculatively to be contributed by high crude fat content and high biological value proteins in buckwheat seeds (*28*). In contrast, group BSP-2 prominently revealed a much higher percent feed efficiency (7.2%) ($p < 0.01$) (**Table 3**), implicating a better intertransformation efficiency among nutrients that had occurred in buckwheat sprout meals. For evidence, the liver/body (L/B) ratios in all sprout meal groups were effectively controlled within a range of 4.7−4.8 g (**Table 3**). Obviously, buckwheat sprouts nutraceutically revealed better healthy bioactive functions than buckwheat seeds in this regard.

Hypolipidemic Activity of Buckwheats. With regard to serum cholesterol levels, all BS and BSP meals revealed a significant cholesterol-lowering effect ($p < 0.05$ for BS1 and $p < 0.01$ for BS2 and BSP groups) (**Table 4**). Kayashita et al. pointed out that buckwheat protein lowers plasma cholesterol and raises fecal sterols in rats fed with cholesterol-enriched diets (*2*). Park et al. found in the rat model that rutin is capable of reducing serum cholesterol, triglycerides, and oxidative product levels by effectively inhibiting the activity of acyl-CoA:cholesterol acyltransferase (ACAT) (*29*). As can be

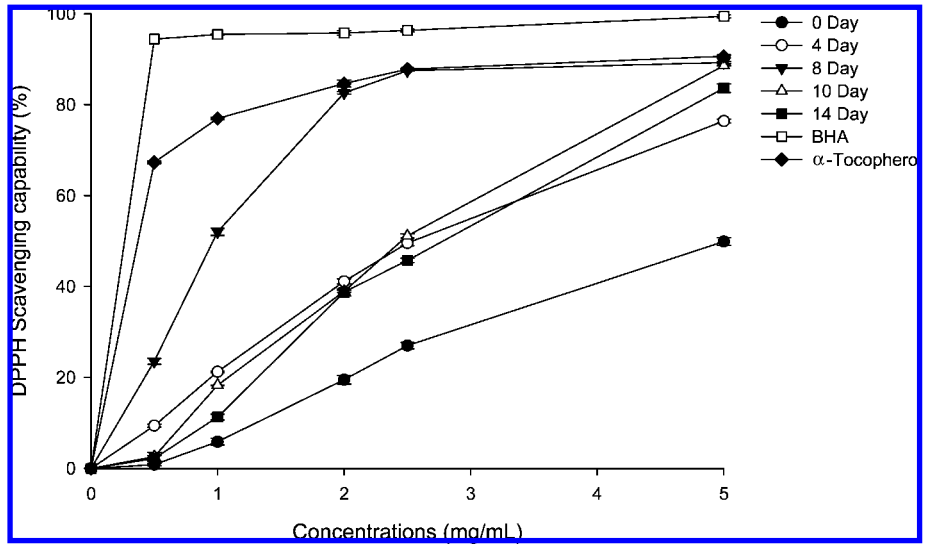

Figure 2. Free-radical scavenging capability on DPPH free radicals by ethanol extracts of dried buckwheat sprouts obtained from different sprouting time. A total of 4 mL of BEE (0.5–20 mg/mL) obtained from different sprouting time was mixed with 1 mL of freshly prepared methanol solution of DPPH free radicals. The mixture was left to stand in the dark for 30 min. The optical density was measured at 517 nm using the Hitachi U-2001 spectrophotometer. α-Tocopherol and BHA were used as positive controls.

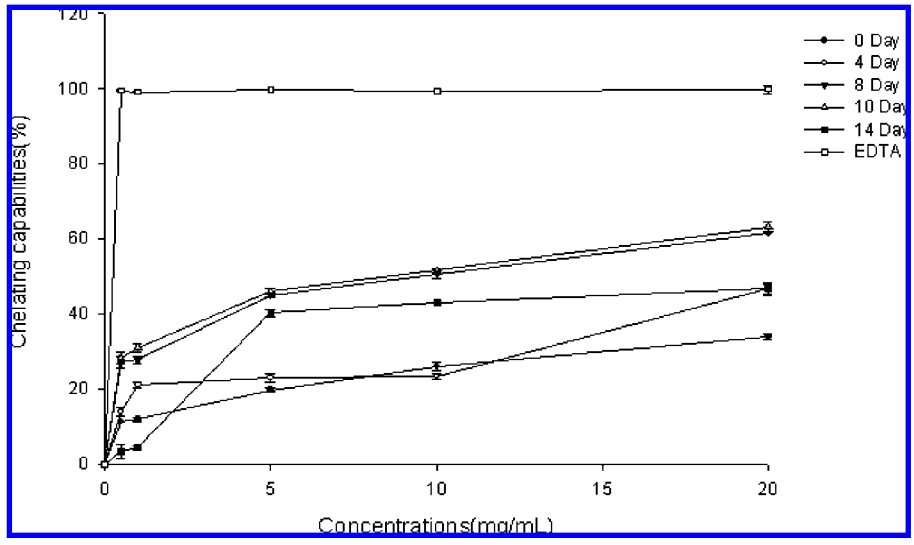

Figure 3. Ferrous ion chelating capabilities by ethanolic extracts of dried buckwheat sprouts obtained from different sprouting time. A total of 1 mL of BEE (0.5–20 mg/mL) was added with methanol (3.7 mL) and ferric chloride solution (2 mM, 0.1 mL). The mixture was left to stand for 30 s to facilitate the reaction. Ferrozine (5 mM, 0.2 mL) was added. The reaction proceeded for 10 min. After the reaction, the optical density was taken immediately, with a spectrophotometry at 562 nm. EDTA was used as a positive control.

expected, the highest value of TG was found in group H, which was consistent with Sessions et al. (*30*). Sprout feeding suppressed serum triglyceride levels in all BS and BSP groups ($p < 0.05$), however, only significantly in groups BS2 and BSP-2 (**Table 4**). The paper by Park et al. further supported such an effect (*29*).

Furthermore, all BS and BSP meals effectively suppressed serum LDL-C ($p < 0.01$). Nutraceutically more important is that serum HDL-C contents were conversely elevated by all BS and BSP meals (**Table 4**). Consequently, ratios of LDL-C/HDL-C were greatly reduced by BS and BSP meals (**Table 4**); such an effect can be very beneficial to the prevention of atherosclerosis (*31*). Moreover, the TC/HDL-C ratios were maintained almost constant at 1.5 in groups BS1, BSP-1, and BSP-2 and slightly higher in group BS2 (**Table 4**), evidence again that buckwheat sprouts could be effective cardiovascular disease preventives (*31*).

Hepatic Lipids. Hepatic cholesterol levels were significantly suppressed by buckwheat feeding. TC content of group H was significantly suppressed in all BS and BSP groups ($p < 0.05$; for BSP-2, $p < 0.01$) (**Table 4**). With a reason yet unclear, the hepatic TG values were totally unaffected by any meal tested (**Table 4**). Odbayar et al. demonstrated effects of some phenolic compounds on lipogenesis in mice (*32*). Quercetin significantly reduced the activity and mRNA levels of various enzymes involved in hepatic fatty acid synthesis. Rutin reduced only a few of the parameters for lipogenesis, suggesting that a reduction in hepatic lipogenesis is the mechanism underlying the hypolipidaemic quercetin. Because rutin appeared more abundant than quercetin in buckwheat sprouts, a failure in reducing fatty acid synthesis was thus perceivable (**Table 2**). Conversely, Park et al. found in the rat model that rutin was capable of reducing plasma cholesterol (*29*), triglycerides, and oxidative product levels, although it was not very effective in suppressing the

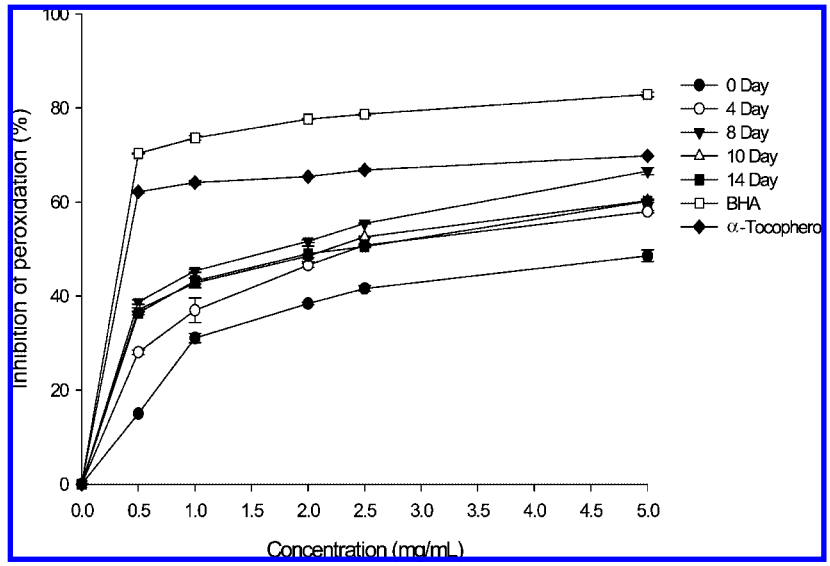

Figure 4. Antioxidant capabilities exhibited by ethanolic extracts of dried buckwheat sprouts obtained from different sprouting times. The reaction mixture consisted of 2 mL of lipid micellar solution (lecithin in MPA buffer), 0.1 mL of $FeCl_3$ solution (25 mM), 0.1 mL of ⁄-ascorbic acid (25 mM), and 1.2 mL of MPA (20 mM, pH 7.4). To the reaction mixture, 0.5 mL of different concentrations of BEE (0.5–20 mg/mL) were added. The mixture was incubated at 37 °C for 2 h. BHT (1 mL, 20 mg/mL), TBA (2 mL, 1%), and TCA (1 mL, 2.8%) were added. A blank was performed in parallel by replacing a same amount of MPA instead of BEE. The reaction mixture was heated on a water bath (100 °C) for 20 min. Upon cooling, the optical density was measured at 532 nm. α-Tocopherol and BHA were used as the positive controls.

Table 3. Changes in Body Weight, Feed Efficiency, Liver Weights, and Ratio of the Liver/Body Weight of Hamsters Fed on Different Meals[a]

	group					
	C	H	BS1	BS2	BSP-1	BSP-2
initial body weight (g)	90 ± 2	88 ± 3	89 ± 6	93 ± 5	89 ± 5	87 ± 2
final body weight (g)	99 ± 4	105 ± 4	101 ± 7	116 ± 7*	102 ± 5	107 ± 5
daily weight gain (g)	0.2 ± 0.1	0.4 ± 0.2	0.3 ± 0.1	0.6 ± 0.2*	0.3 ± 0.1	0.5 ± 0.1*
daily food intake (g)	6.6 ± 0.4	6.0 ± 0.7	6.1 ± 0.7	6.5 ± 0.5*	5.9 ± 1.0	5.9 ± 0.2
percent feed efficiency	4.5 ± 0.4	5.8 ± 0.2	5.1 ± 0.4	5.1 ± 0.2*	5.3 ± 0.2	7.2 ± 0.2**
liver weight (g)	3.3 ± 0.2**	5.5 ± 0.1	5.3 ± 0.4	5.5 ± 0.4	5.0 ± 0.3*	5.0 ± 0.4*
L/B ratio (%)	3.4 ± 0.2**	5.3 ± 1.0	5.2 ± 0.3	4.8 ± 0.2*	4.9 ± 0.3*	4.7 ± 0.3*

[a] Three individual experiments were performed. Values are expressed as mean ± SD ($n = 6$). (*) Significantly different ($p < 0.05$) when compared by Duncan's multiple range tests. (**) Significantly different ($p < 0.01$) when compared by Duncan's multiple range tests. L/B ratio = percent liver/body weight ratio; (liver weight/body weight) × 100%. C, normal formula; H, high fat and cholesterol meal; BS-1, high fat and cholesterol meal containing 2.5% buckwheat seeds; BS-2, high fat and cholesterol meal containing 25% buckwheat seeds; BSP-1, high fat and cholesterol meal containing 2.5% buckwheat sprouts; and BSP-2, high fat and cholesterol meal containing 25% buckwheat sprouts.

Table 4. Serum and Hepatic Lipidaemic Parameters in Hamsters Fed on Different Buckwheat Meals[a]

	group					
parameters	C	H	BS1	BS2	BSP1	BSP2
serum (mg/dL)						
TC	121 ± 16**	217 ± 20	181 ± 11*	179 ± 10**	170 ± 8**	161 ± 8**
TG	69 ± 15**	112 ± 12	103 ± 21	96 ± 33*	99 ± 21	96 ± 17*
LDL-C	64 ± 4**	95 ± 6	75 ± 10**	75 ± 9**	62 ± 5**	61 ± 5**
HDL-C	92 ± 14*	108 ± 19	119 ± 9.5	109 ± 11	116 ± 11	110 ± 14
LDL-C/HDL-C ratio	0.7 ± 0.1**	0.9 ± 0.2	0.6 ± 0.0**	0.7 ± 0.0**	0.5 ± 0.0**	0.6 ± 0.1**
TC/HDL-C ratio	1.3 ± 0.1**	2.0 ± 0.1	1.5 ± 0.1**	1.6 ± 0.0**	1.5 ± 0.0**	1.5 ± 0.0**
hepatic (mg/g)						
TC	17 ± 1**	20 ± 1.0	18 ± 1*	18 ± 1*	18 ± 0*	17 ± 1**
TG	22 ± 1**	26 ± 2	25 ± 1	25 ± 1	25 ± 1	25 ± 1

[a] TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol. Three individual experiments were performed. Values are expressed as mean ± SD ($n = 6$). (*) Significantly different ($p < 0.05$) when compared by Duncan's multiple range tests. (**) Significantly different ($p < 0.01$) when compared by Duncan's multiple range tests. C, normal formula; H, high fat and cholesterol meal; BS-1, high fat and cholesterol meal containing 2.5% buckwheat seeds; BS-2, high fat and cholesterol meal containing 25% buckwheat seeds; BSP-1, high fat and cholesterol meal containing 2.5% buckwheat sprouts; and BSP-2, high fat and cholesterol meal containing 25% buckwheat sprouts.

activity of liver HMG-CoA reductase yet effectively inhibited the activity of ACAT. Buckwheat sprouts and seeds contain profound amount of fibers, which accelerate bile acid secretion and metabolism. Alternatively, sprouting increases fiber content (data not shown). Fibers form conjugates with bile acids, excreted in feces, and reduce the recycled bile acid metabolites. Through such a mechanism, the plasma cholesterol level can be greatly reduced. Results are rather consistent with Kayashita et al. and Glore et al. (*2, 34*). When the results are taken together, the hypocholesterolemic effect exerted by day 8 buckwheat

sprouts can be attributed to the synergistic effect exerted by high crude fiber contents and high rutin and quercetin concentrations in day 8 sprouts, which lead to the potential suppression of cholesterol *de novo* synthesis. More importantly, the acute toxicity test performed for 7 days by the Japan Food and Safety Examination Bureau confirmed the nontoxic characteristics of 25% dried-sprout-containing meals (data not shown).

To summarize, day 8 buckwheat sprouts possess maximum levels of nutrients and bioactivities, which exhibit the most active antioxidative and free-radical scavenging capabilities. More importantly, its potent hypocholesterolemic and hypotriglyceridemic activities when tested by the Syrian hamster mode can be ascribed to its surprisingly high polyphenolic and moderate quercetin contents.

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Received for review September 29, 2007. Revised manuscript received November 1, 2007. Accepted November 1, 2007.

JF072886X