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Effect of Buckwheat Extract on the Antioxidant Activity of Lipid in Mouse Brain and Its Structural Change during in Vitro Human Digestion

Sun-Jin Hur,[†] Soo-Jeong Park,[‡] and Chang-Ho Jeong[*,§]

[†]Department of Applied Biochemistry, Konkuk University, Chungju 380-701, Korea

[‡]Department of Horticulture, Institutes of Agriculture and Life Sciences, Gyeongsang National University, Jinju 660-701, Korea

[§]Research Center, Wooyang Frozen Food Company, Ltd., Seocheon 325-907, Korea

ABSTRACT: This study was conducted to investigate the effects of buckwheat (*Fagopyrum esculentum* Moench cv. Yangjul No. 2) extract on the antioxidant activity of lipids in mouse brain and the structural change during in vitro human digestion. Buckwheat was collected from a wild farm and extracted with water. The buckwheat extracts were then passed through an in vitro human digestion model that simulated the composition of the mouth, stomach, and small intestine juice. The results confirmed that the main phenolics of buckwheat extract were rutin, quercitrin, and quercetin. The rutin content increased with digestion of the buckwheat (from 48.82 to 96.34 μg/g) and rutin standard samples (from 92.76 to 556.56 μg/g). Antioxidant activity was more strongly influenced by in vitro human digestion of both buckwheat and rutin standard. After digestion by the small intestine, the antioxidant activity values were dramatically increased (from 5.06 to 87.82%), whereas the antioxidant activity was not influenced by digestion in the stomach for both buckwheat extract and rutin standard. Inhibition of lipid oxidation of buckwheat in mouse brain lipids increased after digestion in the stomach for both buckwheat extract and the rutin standard. The major finding of this study was that in vitro human digestion may be an important modulator of the antioxidant capacity of buckwheat and that this may be because in vitro human digestion increased the antioxidative activity via an increase in antioxidants such as rutin and quercetin.

KEYWORDS: buckwheat, antioxidant activity, mouse brain lipid, in vitro human digestion

INTRODUCTION

Buckwheat bears a triangular seed with a hull covering the light green to white kernel, and the main form of buckwheat consumed is seed.[1] Buckwheat seed contains antioxidants such as rutin, tocopherols, and phenolic acid;[2] buckwheat is especially recognized as a plant rich in rutin and quercetrin.[3] Rutin, a natural flavone derivative, was first discovered in buckwheat in the 19th century and is a common dietary flavonoid that is found in fruits, vegetables, and plant-derived beverages such as tea and wine.[4] Several studies[5−7] have reported that whole buckwheat contains 2−5 times more phenolic compounds than oats or barley and that bran and hulls have 2−7 times higher antioxidant activity than barley, triticale, and oats. Therefore, consumption of large amounts of buckwheat bran is considered to have significant nutritional or medicinal benefits.[8]

Several studies have reported that fruits and vegetables may play an important role in delaying the onset of Alzheimer's disease, particularly among those who are at high risk for the disease.[9,10] Thus, high vegetable consumption may also be associated with slower rate of cognitive decline in older age,[10,11] and consumption of phytochemicals may have important applications in the future as natural antiaging or antioxidant agents for the food industry. Rutin possesses several pharmacological activities including antiallergic, anti-inflammatory, vasoactive, antitumor, antibacterial, antiviral, and antiprotozoal properties and is widely used in treating diseases.[12]

Several studies[13−15] have also reported the effect of various phytochemicals on antioxidant activity. However, the effect of phytochemicals on the antioxidant activity of lipid in mouse brain has not been studied in buckwheat, nor have the effects of in vitro human digestion on the structural changes and antioxidant activity in buckwheat. Moreover, there are a limited number of studies concerning the effect of food components on buckwheat's intestinal absorption despite the importance for judging bioavailability. Therefore, the purpose of this study was to determine the effect of buckwheat extracts on the antioxidative activity of lipid in mice brain and its structural changes during in vitro human digestion.

MATERIALS AND METHODS

Materials. Potassium chloride, potassium hydroxide, potassium persulfate, sodium sulfate, sodium hydrogen carbonate, hydrogen chloride, potassium phosphate monobasic, magnesium chloride, hexane, methanol, acetate, thiobarbituric acid, trichloroacetic acid, phosphoric acid, ferric chloride, hydrochloric acid, ether, and ethanol were purchased from Fisher Scientific Chemical Co. (Pittsburgh, PA). Bicarbonate, potassium thiocyanate, sodium phosphate dibasic, sodium phosphate monobasic, sodium chloride, calcium chloride, ammonium chloride, urea, glucose, glucuronic acid, glucoseamine, α-amylase, uric acid, mucin, bovine serum albumin, pepsin, pancreatin, lipase, bile salt extraction, butylated hydroxyanisole, phenolphthalein, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), L-ascorbic acid, malondialdehyde, quercitrin, quercetin, and rutin were purchased from

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Table 1. Constituents and Concentrations of the Various Synthetic Juices of the in Vitro Human Digestion Model[a]

	saliva	gastric juice	duodenal juice	bile juice
organic + inorganic solution	290 mg of α-amylase	1 g of bovine serum albumin	9 mL of $CaCl_2 \cdot 2H_2O$ 22.2 g/L	10 mL of $CaCl_2 \cdot 2H_2O$ 22.2 g/L
	15 mg of uric acid	2.5 g of pepsin	1 g of bovine serum albumin	1.8 g of bovine serum albumin
	25 mg of mucin	3 g of mucin	9 g of pancreatin	30 g of bile
			1.5 g of lipase	
pH	6.8 ± 0.2	1.30 ± 0.02	8.1 ± 0.2	8.2 ± 0.2

[a] The inorganic and organic solutions are augmented to 500 mL with distilled water. If necessary, the pH of the juices is adjusted to the appropriate interval.

Sigma-Aldrich Chemical Co. (St. Louis, MO). Bis[trimethylsilyl]-trifluoroacetamide and trimethylchlorosilane were purchased from Supelco Co. (St. Louis, MO).

In Vitro Human Digestion Model. The in vitro human digestion model used was a modified version of that described in previous studies:[16,17]

I *Pre-ingestion* comprised the dried buckwheat extract and rutin standard.

II *Mouth*: About 1 g of dried buckwheat extract and rutin standard were mixed with 6 mL of simulated saliva fluid (pH 6.8) and then stirred for 5 min at 37 °C.

III *Stomach*: About 12 mL of simulated gastric fluid (pH 2) was added, and then the mixture was stirred for 2 h at 37 °C.

IV *Small Intestine*: About 12 mL of duodenal juice, 6 mL of bile juice, and 2 mL of bicarbonate solution (pH 6.5−7) were added, and then the mixture was stirred for 2 h at 37 °C.

The compositions of the simulated saliva, gastric, duodenal, and bile fluids are listed in Table 1. During the in vitro human digestion model the samples were swirled (60 rpm) on a shaking water bath to simulate the motility of the gastrointestinal tract (model HB-205SW, Hanbaek, Co., Bucheon, Korea).

Plant Materials. Buckwheat (*Fagopyrum esculentum* Moench cv. Yangjul No. 2) grain was collected from a wild buckwheat farm in Gangwondo Agricultural Research and Extension Services (Pyeongchang, Korea) and then dried at room temperature. Dried buckwhaeat grain was then ground into a fine powder and stored in sealed polyethylene bags at −20 °C until analysis. Rutin standard (Sigma-Aldrich Chemical Co.) was also used as a positive control. Both samples were passed through the in vitro human digestion model, and their enzyme activities after each step were compared.

Extraction of Buckwheat. Aqueous extracts of freeze-dried buckwheat grain powder were obtained as follows. Buckwheat (100 g) grain powder was suspended and extracted with 2 L of water at 100 °C for 2 min. The extracts were filtered through Whatman no. 2 filter paper and evaporated to dryness. The aqueous extract was concentrated in a vacuum evaporator at 40 °C. Water filtrate was frozen and lyophilized. The extracts were placed in a glass bottle and stored at −20 °C until used. The lyophilized extracts were redissolved in water to a concentration of 1000 μg/mL.

ABTS Radical-Scavenging Activity. ABTS was dissolved in water to make a concentration of 7 mmol/L. ABTS was produced by reacting the ABTS stock solution with 2.45 mmol/L potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12−16 h before use. For the test of samples, the ABTS stock solution was diluted with phosphate-buffered saline 5 mmol/L (pH 7.4) to an absorbance of 0.70 at 734 nm. After the addition of 980 μL of diluted ABTS to 20 μL of sample, the absorbance reading was taken 5 min after the initial mixing.[18] This activity is given as percent ABTS scavenging that is calculated as

$$\%\ \text{ABTS scavenging activity} = [(\text{control absorbance} - \text{sample absorbance})/\text{control absorbance}] \times 100$$

Ferric Reducing Antioxidant Power (FRAP) Assay. This study employed the FRAP assay developed in a previous study.[18] In short, 1.5 mL of working, prewarmed 37 °C FRAP reagent (10 vol of 300 mmol/L acetate buffer, pH 3.6, plus 1 vol of 10 mmol/L 2,4,6-tripyridyl-*S*-triazine in 40 mmol/L hydrochloric acid plus 1 vol of 20 mmol/L ferric chloride) was mixed with 50 μL of test samples and standards. This was vortex mixed, and the absorbance at 593 nm was read against a reagent blank at a predetermined time after sample−reagent mixing. The test was performed at 37 °C, and the 0−4 min reaction time window was used.

Lipid Oxidation in Mouse Brain Lipid. Twenty Balb/C mice (4 months old, average 50 ± 5 g body weight) were obtained from Dr. Jeong's laboratory in Gyeongsang National University. After a 1 week adaption, the mice were sacrificed by CO_2 gas. The skull was opened and brains were collected. Malondialdehyde (MDA) assay as a lipid oxidation value was carried out according to the method described in a previous study.[19] Collected brains were homogenized in ice-cold Tris-HCl buffer (20 mM, pH 7.4) to produce a $^1/_{10}$ homogenate. The homogenate was centrifuged at 12000*g* for 15 min at 4 °C. Aliquots (0.1 mL) of the supernatant were incubated with the test samples (0.2 mL) in the presence of 10 μM ferrous sulfate (0.1 mL) and 0.1 mM vitamin C (0.1 mL) at 37 °C for 1 h. The reaction was terminated by the addition of 0.1 mL of trichloroacetic acid (28%, w/v) and 0.3 mL of thiobarbituric acid (1%, w/v) in succession, and then the solution was heated at 100 °C. After 15 min, the color of the MDA−thiobarbituric acid complex was measured at 532 nm. (+)-Catechin, a well-known antioxidant, was used as a positive control. The inhibition ratio (%) was calculated as follows: % inhibition = [(control absorbance − sample absorbance)/control absorbance] × 100. Figure 1 shows the process of lipid oxidation analysis in mouse brain lipid.

Quantification of Phenolics by High-Performance Liquid Chromatography (HPLC). Rutin, quercitrin, and quercetin in phenolic extract from buckwheat were measured at 280 nm using rutin standard solution by a diode array ultraviolet−visible detector (Agilent 1100 series, Agilent Co., Santa Clara, CA). Separation was achieved with a Shiseido C18 column (250 mm × 4.6 mm i.d., 5 μm, Shiseido Co., Tokyo, Japan). The elution solvents were (A) 0.01 M potassium phosphate buffer adjusted to pH 3.0 by phosphoric acid and (B) methanol. The solvent gradient elution program used was as follows: initial 90% (A), hold for 9.5 min; linear gradient to 68% (A) in 3.5 min; linear gradient to 67% (A) in 17 min; linear gradient to 20% (A) in 1 min; linear gradient to 90% (A) in 1 min, and hold for 10 min. The flow rate was 1.5 mL/min. Rutin was identified by comparison of their retention time values and ultraviolet spectra with those of known standards and quantified by peak areas from the chromatograms. All analyses were run in quintuplicate, and mean values were calculated. Contents of rutin, quercitrin, and quercetin were expressed in micrograms per gram of extract.

Statistical Analysis. The data were analyzed using SAS software (SAS Institute Inc., Cary, NC; 2001) by the generalized linear model procedure. The Student−Newman−Keuls' multiple-range test was used to compare differences among means. Significance was defined at $P < 0.05$.

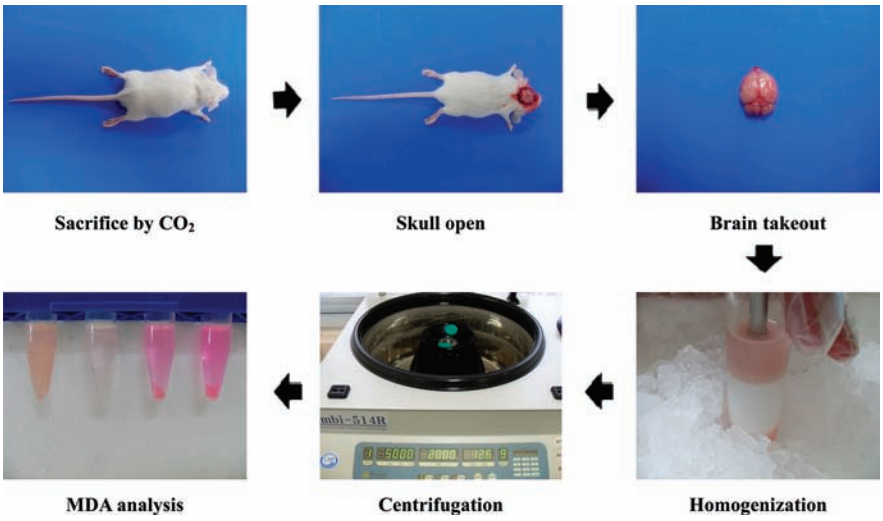

Figure 1. Process of lipid oxidation analysis in mouse brain lipid.

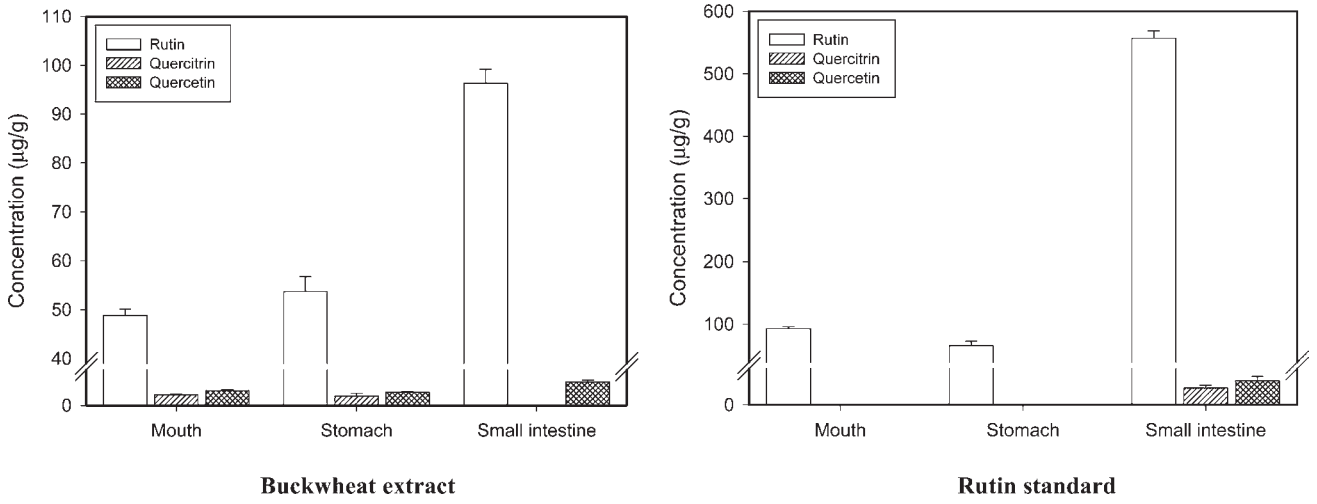

Figure 2. Rutin, quercitrin, and quercetin contents of phenolic extract from buckwheat as they pass through an in vitro human digestion model.

RESULTS AND DISCUSSION

The phenolics contents of the buckwheat extract and rutin standard during in vitro human digestion are shown in Figure 2. The main phenolics of the buckwheat extract were rutin, quercitrin, and quercetin. The amount of rutin increased dramatically after digestion in the small intestine for both buckwheat extract (from 48.82 to 96.34 μg/g) and rutin standard (from 92.76 to 556.56 μg/g); however, the quercitrin content decreased from 2.36 μg/g to trace with digestion steps for buckwheat extract and increased from trace to 5.28 μg/g with digestion steps for rutin standard. The antioxidant activity of buckwheat extract and rutin standard during in vitro human digestion is presented in Figure 3. In the present study, the ABTS and FRAP methods were used to determine the antioxidant activity. After digestion in the small intestine, the antioxidant activity values of the buckwheat extract and rutin standard increased dramatically on the basis of both ABTS (from 5.06 to 87.82%) and FRAP analyses (0.56 to 0.90 OD), whereas the antioxidant activity was not influenced until digestion in the stomach for both buckwheat extract and rutin standard. The inhibitory effect of lipid oxidation in buckwheat extract in mouse brain lipids is presented in Figure 4. Inhibition of lipid oxidation of buckwheat extract increased after digestion in the stomach for both buckwheat extract and rutin standard.

In the present study, we simulated mouth, stomach, and small intestine digestion of buckwheat extract and rutin standard to determine the stability of phytochemicals and antioxidant activity under in vitro human digestion conditions. The results of the present study revealed that the amount of phenolic compounds such as rutin and quercetin increased during in vitro human digestion (after digestion by the small intestine) and that the antioxidant activity of buckwheat extract increased as the level of phenolic compounds increased. These findings indicated that the antioxidant activity increased in response to digestion. Moreover, the lipid oxidation inhibition effect of buckwheat extract in mouse brain lipids also increased as the level of phenolic compounds increased. Thus, we assumed that in vitro human digestion increased the antioxidant activity.

Buckwheat is recognized as a plant rich in rutin and quercetrin.[3] Rutin is a flavonol glycoside plant metabolite with

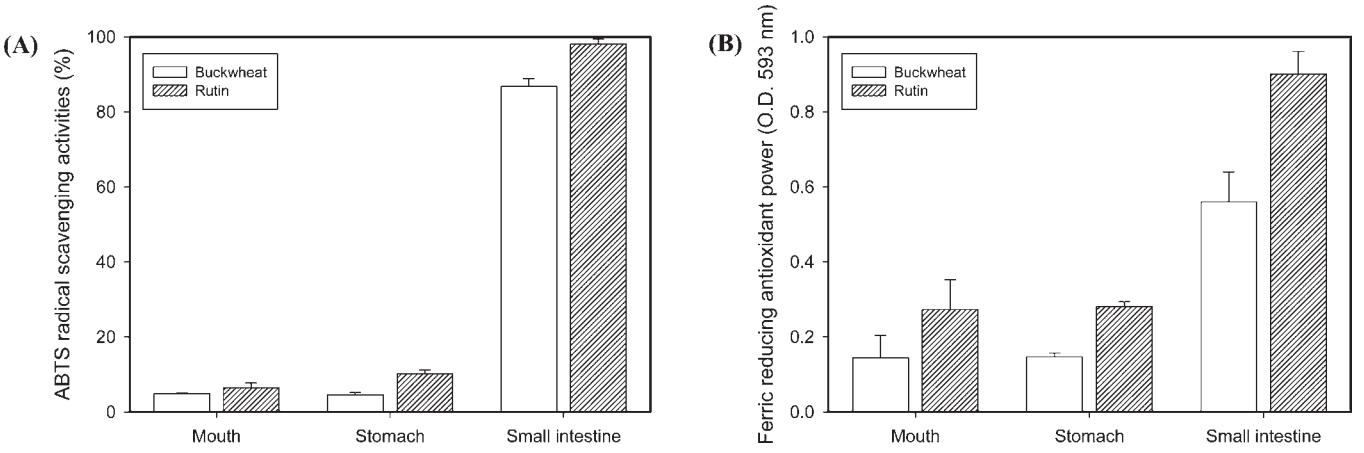

Figure 3. ABTS (A) and FRAP (B) of extract from buckwheat and rutin standard as they pass through an in vitro human digestion model. Mouth step, saliva juice after 5 min; stomach step, gastric juice after 2 h; small intestine step, duodenal juice and bile juice after 2 h. Results are presented as the mean ± SD of three independent experiments conducted in triplicate. Different letters are significantly different at $P < 0.05$.

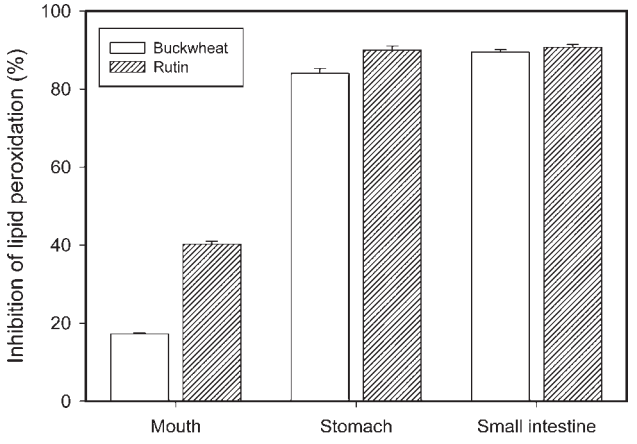

Figure 4. MDA of extract from buckwheat and rutin standard as they pass through an in vitro human digestion model. Mouth step, saliva juice after 5 min; stomach step, gastric juice after 2 h; small intestine step, duodenal juice and bile juice after 2 h. Results are presented as the mean ± SD of three independent experiments conducted in triplicate. Different letters are significantly different at $P < 0.05$.

antioxidative, anti-inflammatory, and anticarcinogenic effects.[20] In the present study, the amount of rutin present in buckwheat extract increased after in vitro human digestion by the small intestine. This may have been due to rutin isolation from buckwheat being increased by in vitro digestion. Most dietary polyphenols are quite stable during gastric digestion.[21] Conversely, dietary polyphenols are highly sensitive to the mild alkaline conditions in the small intestine; therefore, during digestion in the duodenum a portion of these compounds may be transformed into different structural forms with different chemical properties.[21] The pH shift to >7.5 during pancreatic/small intestine digestion was the primary factor involved in the irreversible breakdown of the anthocyanins.[22] In the present study, the increase in rutin after in vitro human digestion by the small intestine may have been due to the difference in pH between the stomach and small intestine. Other factors such as oxygen, enzymes, and temperature will also affect the phenolic compositions.[23] Another possible mechanism for increasing the antioxidant activity of buckwheat extract is that the isolation of quercetin from rutin is increased by in vitro human digestion. Quercetin is one of the most common native flavonoids, occurring primarily in glycosidic forms such as rutin.[24] A number of studies have demonstrated that quercetin inhibits lipid peroxidation effectively by scavenging free radicals and/or chelating transition metal ions.[25] The phenolic hydroxyl groups of flavonoids, which act as electron donors, are responsible for the free radical-scavenging activity of the compounds.[25] The antioxidant activity of most flavonoids is closely related to their structure, specifically, the structure and position of the sugar moiety.[26] The presence of vicinyl dihydroxyl groups has been shown to affect the ability of phenols to inhibit iron- and copper-catalyzed production of initiating radical species.[27] Rutin is only slightly weaker than quercetin with respect to its ability to reduce Fe(II)-induced MDA formation in liposomes and ferrous sulfate/cysteine-induced lipid peroxidation in rat liver miscosomes.[28] Thus, the increase in quercetin by in vitro human digestion is likely one of the primary reasons for the increase in the antioxidant activity of buckwheat extract observed in the present study.

A previous study[28] reported that the significantly higher intracellular peroxyl radical-scavenging activity of quercetin could be explained by the difference in cell membrane permeability between quercetin and rutin owing to their different partition coefficients resulting from their structural characteristics. Rutin is less able to penetrate cell membranes because it carries a hydrophilic disaccharide moiety.[28] However, quercetin may diffuse through the cell membrane more efficiently than rutin because it does not carry rutinose, is hydrophilic, and reduces Cu^{2+}-induced oxidative stress by scavenging radicals instead of chelating with metal ions.[28] In the present study, penetration of quercetin in the cell membrane may have increased via in vitro human digestion because the amount of quercetin in the buckwheat extract increased during in vitro human digestion and was the cause of the antioxidant effect in mouse brain lipids. It was previously reported[25] that intact flavonoid glycosides were not well absorbed by the small intestine because sugar moieties elevate their hydrophilicity. Flavonoid glycosides from the diet are believed to pass through the small intestine and then enter the cecum and colon, where they are hydrolyzed to aglycone by enterobacteria.[29] Flavonoid aglycone can be easily absorbed into epithelial cells in the large

intestine because its lipophilicity facilitates its passage across the phospholipid bilayer of cellular membranes.[25] In the present study, antioxidative activity was dramatically increased after digestion by the small intestine. This may have been due to rutin being a flavonoid glycoside that was hydrolyzed to quercetin as a flavonoid aglycone by in vitro human digestion. Previous studies[30] also reported that rutin was not well absorbed when compared with quercetin aglycone because rutin was not easily digested in the small intestine, but was, rather, in the large intestine by intestinal microflora. On the basis of the results of this study, we assume that the quercetin aglycone form had a better antioxidant effect than rutin glycoside during in vitro human digestion. The results of a previous study[31] agree with our suggestion that quercetin aglycone appears to be a more active chain-breaking antioxidant than its glycoside counterparts because of its higher accessibility to the site of chain-initiating and chain-propagating free radicals in membranous phospholipid bilayers.

The quercetin metabolite possesses considerable free radical-scavenging activity because it contains the radical-scavenging catechol group.[32] An earlier study demonstrated that dietary quercetin could accumulate in the human circulatory system.[25] Neither quercetin aglycone nor quercetin glucosides were found to be present in the blood plasma in an experiment using rats.[33] Therefore, ingestion of rutin-rich foods such as buckwheat can lead to increased rutin and quercetin in the circulatory system and increased antioxidant activity. The major finding of this study was that in vitro human digestion may be an important modulator of the antioxidant capacity of buckwheat. This may be due to in vitro human digestion increasing the antioxidant activity by increasing the levels of antioxidants such as rutin and quercetin.

AUTHOR INFORMATION

Corresponding Author

*Phone: 82-41-956-7171. Fax: 82-41-956-7170. E-mail: difco5479@foodkorea.com.

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