Catechins as Antioxidants from Buckwheat (*Fagopyrum esculentum* Moench) Groats

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Four catechins and rutin were isolated from ethanol extracts of buckwheat (*Fagopyrum esculentum* Moench) groats by Sephadex LH-20 column chromatography and preparative HPLC with monitoring of the peroxyl radical scavenging activity. The antioxidant activity of these catechins was higher than that of rutin. The structures of these catechins were established as (−)-epicatechin, (+)-catechin 7-*O*-*β*-D-glucopyranoside, (−)-epicatechin 3-*O*-*p*-hydroxybenzoate, and (−)-epicatechin 3-*O*-(3,4-di-*O*-methyl)gallate on the basis of ^{1}H, ^{13}C, and two-dimensional nuclear magnetic resonance techniques and fast atom bombardment mass spectrometry. The yields of these antioxidant compounds suggest that they are abundant, as is rutin, which is known as a biological phytochemical in buckwheat groats.

Keywords: *Fagopyrum esculentum; antioxidant activity; flavonoids; (−)-epicatechin; (+)-catechin 7-O-β-D-glucopyranoside; (−)-epicatechin 3-O-p-hydroxybenzoate; (−)-epicatechin 3-O-(3,4-di-O-methyl)gallate*

INTRODUCTION

Buckwheat belongs to the Polygonaceae family, unlike major cereals such as wheat, rice, and corn. This family contains useful plants, for example, rhubarb (*Rheum officinale*, *R. palmatum*, *R. coreanum*), which has use as a medicinal plant, and the Japanese indigo plant, ai (*Polygonum tinctorium* Ait.), which is used as a dye. Recently buckwheat has been recognized as a healthy food because its seed is rich in vitamins B_1 and B_2, its protein has high biological value (Sure, 1955), it has valanced amino acid composition, it is rich in lysine (Pomeranz and Robbins, 1972), and, especially, it contains rutin, which has a hypotensive effect (Matsubara et al., 1985) and strengthens the capillary blood vessels, resulting in the prevention of cerebral apoplexy. In addition, buckwheat has various advantages also in cultivation, such as a short growing season (70−90 days) and negligible crop protection. For these reasons, buckwheat is cultivated in China, the United States, Canada, Japan, and all parts of the world.

Formerly, we detected antioxidant activity in the ethanol extracts from both buckwheat hulls and groats (Watanabe et al., 1995). The antioxidant activity of the extract from the hulls was attributed to phenolic compounds including flavonoids (Watanabe et al., 1997). Although some flavonoids have been detected along with rutin in buckwheat seedlings (Margna and Margna, 1982), isolated from its immature seeds (Sato and Sakamura, 1975), and detected in mature seeds (Luthar and Kreft, 1996), so far, no studies have tried to investigate the antioxidant compounds in its groats. In this paper, we have isolated and identified antioxidant compounds from buckwheat groats to obtain information in relation to their biological activity.

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MATERIALS AND METHODS

Materials. Buckwheat (*Fagopyrum esculentum* Moench cv. Iwate zairai) seeds were harvested in Tohoku National Agricultural Experiment Station in 1993, dried with ventilation at ambient temperature, and stored at 4 °C until use. The seeds were dehulled by a grinder, and the hulls were removed by winnowing. The dehulled buckwheat groats were manually separated from their intact kernels.

Chemicals. Catechins [(+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate] were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Sugars [D-(+)-glucose, D-(+)-galactose, and L-(+)-rhamnose] and BHA were purchased from Nacalai Tesque Inc., Ltd. (Kyoto, Japan). Quercetin was obtained from Extrasynthèse S.A. (Genay, France). Methyl linoleate was obtained from Tokyo Kasei Organic Chemicals Co., Ltd. (Tokyo, Japan), and it was purified as previously described (Terao and Matsushita, 1986). 2,2′-Azobis(2,4-dimethylvaleronitrile) and tannase were obtained from Wako Pure Chemicals Industries (Osaka, Japan). Trimethylchlorosilane, hexamethyldisilazane, and the solvents for NMR analysis were purchased from Merck (Darmstadt, Germany).

Extraction. The procedure for the isolation of antioxidant compounds from buckwheat groats is shown in Figure 1. The groats were ground by a vibrating sample mill, and the flour (300 g) was extracted with 6 L of ethanol under reflux in a water bath at 80 °C for 1 h. The sample was filtered with Advantec no. 5C (Toyo Inc.) filter paper and concentrated under reduced pressure at 40 °C. The concentrated filtrate was used as the crude extract for the experiment.

Sephadex LH-20 Column Chromatography. The crude extract was separated on a Sephadex LH-20 (Pharmacia Co., Ltd.) column (46 × 730 mm i.d.) by stepwise elution with ethanol and methanol, with monitoring at 280 and 350 nm. Seven fractions (A−G) were obtained from the ethanolic eluate, and the methanolic eluate was collected as one fraction (H). All of the fractions were concentrated under reduced pressure at 40 °C. Antioxidant activity was present in fractions B and D−H. Fraction F with the highest antioxidant activity and fraction E, the HPLC chromatogram of which indicated the presence of rutin, were used for the isolation of the antioxidant compounds.

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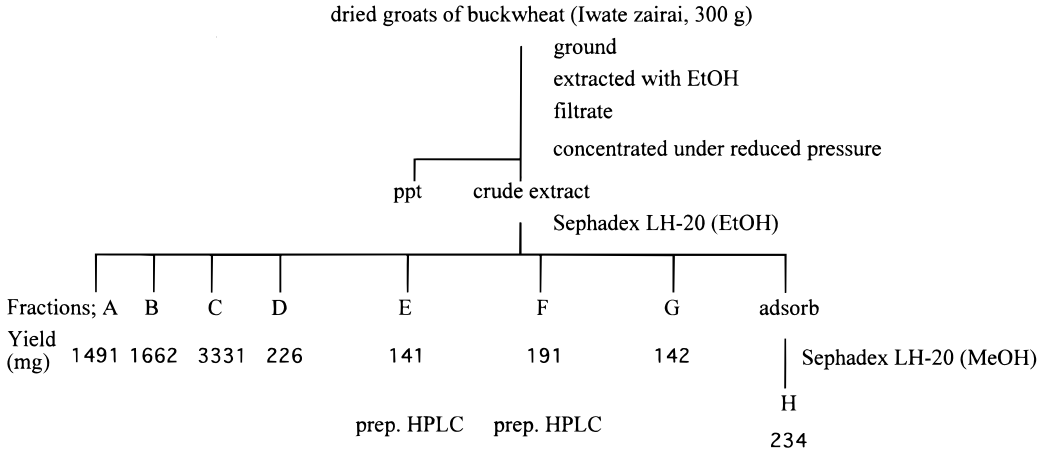

Figure 1. Scheme for preparation of antioxidant compounds from buckwheat groats and yields of fractions separated by Sephadex LH-20 column chromatography.

Preparative HPLC. Two major peaks in the HPLC chromatogram of fraction E and four major ones in that of fraction F were isolated by preparative HPLC fitted with an ODS column. Conditions were as follows: column, Cosmosil 5C18 (Nacalai Tesque, Inc., Ltd., 20 × 250 mm i.d.); mobile phase, linear gradient of methanol/water (containing 2.5% acetic acid); 5−59% methanol over 48 min; flow rate, 7 mL/min; detection, UV detector (166 type, Beckman Co., Ltd.; 280 nm). Each compound collected was concentrated at 40 °C under reduced pressure. Their yields were the following: compound $\mathbf{E_1}$, 3.5 mg; $\mathbf{E_2}$, 26.1 mg; $\mathbf{F_1}$, 30.4 mg; $\mathbf{F_2}$, 7.7 mg; $\mathbf{F_3}$, 3.6 mg; $\mathbf{F_4}$, 26.6 mg.

Analytical HPLC. To check the purity of the isolated compounds and their identification, they were analyzed by HPLC equipped with a photodiode array detector. The conditions of the HPLC were as follows: column, Cosmosil 5C18 (Nacalai Tesque Inc., Ltd., 4.6 × 250 mm i.d.); mobile phase, linear gradient of methanol/water (containing 2.5% acetic acid), 23−50% methanol over 40 min for compounds derived from fraction E, 5−95% methanol over 30 min for compounds derived from fraction F; flow rate, 1 mL/min; detection, photodiode array detector (168 type, Beckman Co., Ltd.; on-line acquisition of absorption spectra, 240−400 nm).

TLC. The fractions separated by Sephadex LH-20 and isolated compounds were characterized by TLC under two conditions: (A) for the analysis of phenolic compounds; (B) for lipids.

(a) Condition A. The fractions and isolated compounds were examined on cellulose TLC plates (2−15 μm particle size, 0.1 mm thickness; Merck) using 1-butanol/acetic acid/water (BAW, 4:1:5 v/v/v) and, subsequently, 30% acetic acid. The chromatograms were visualized by UV with ammonia vapor and UV after spraying with a vanillin−HCl reagent. The red color of the spots of the compounds derived from fraction F in the chromatogram with the vanillin−HCl reagent suggested that these compounds contained flavan-3-ol, catechin, structure. Their structures were elucidated on the basis of the data of NMR spectrometer compared with authentic standard catechins.

(b) Condition B. The composition of lipids in fractions was examined on silica gel 60 TLC plates (5−20 μm mean particle size, 0.25 mm thickness; Merck) using *n*-hexane/ether (95:5 v/v). The chromatograms were left in an oven at 130 °C for 5 min after spraying with 50% sulfuric acid.

Instrumental Analysis. *(a) Fast-Atom Bombardment Mass Spectrometry (FAB-MS).* The positive ion mode of the FAB-MS spectra of the isolated compounds was recorded on a JEOL JMS-AX505H mass spectrometer, which was operated at a 10-kV accelerating potential. The samples were mixed with glycerol as the mounting matrix and ions were produced by bombardment with Ar atoms of 6-kV energy.

(b) ^1H and ^{13}C NMR. ^1H (399.65 MHz) and ^{13}C NMR (100.40 MHz) spectra of the isolated compounds were recorded on a JEOL JNM-EX 400 spectrometer with DMSO-d_6 at 40 °C or acetone-d_6/D_2O (1:1) solvent mixture at 30 °C. To determine the chemical shifts given in the δ value (ppm), TMS was used as an internal standard with DMSO-d_6, and peaks of δ 2.05 in ^1H NMR and δ 29.8 in ^{13}C NMR in acetone-d_6 were used as the reference peaks.

(c) 2D NMR Spectra. To confirm the assignment of the NMR spectrum of the isolated compounds, $^{13}C-^1H$ correlation spectroscopy (COSY) was measured with J_{CH} = 140 Hz, and correlation spectroscopy via long-range coupling (COLOC) was measured with J_{CH} = 8 Hz. DMSO-d_6 was used as the solvent.

Acid Hydrolysis of F_1 and Sugar Analysis of TMS Derivatives by Gas Chromatography (GC). The result of the ^1H and ^{13}C NMR spectra of compound $\mathbf{F_1}$ suggested that this compound contained one molecule of sugar moiety. To confirm the type and linkage of sugar residue, namely, either *C*-glycoside or *O*-glycoside, compound $\mathbf{F_1}$ was hydrolyzed under acidic conditions in the following manner: To 1.0 mg of the compound was added MeOH (2.5 mL) and 2 N HCl (2.5 mL). This mixture was heated in a water bath at 100 °C under reflux for 60 min and washed with ethyl acetate (3 × 4 mL). The H_2O fraction was evaporated to dryness under reduced pressure at 40 °C. The dried H_2O fraction was trimethylsilylated by reacting 0.25 mg of the residue of the fraction with hexamethyldisilazane (0.05 mL) in dry pyridine (0.1 mL). Trimethylchlorosilane (0.05 mL) was then added and left for 30 min. This mixture was then evaporated to dryness under reduced pressure at 40 °C. The residue was dissolved in dry heptane (0.1 mL), and this solution was used for the determination of the sugar. The trimethylsilylated derivatives of authentic standards of sugars prepared in the same manner were used as references.

A Hewlett-Packard 6890 gas chromatograph with flame ionization detector (FID) was used for the analysis of trimethylsilylated sugars. The conditions of the GC were as follows: column, fused silica capillary column HP-1701, length = 30 m, film thickness = 0.25 μm, i.d. = 0.32 mm (Hewlett-Packard); oven temperature, 125−250 °C with a linear gradient of 7 °C/min; carrier gas, helium; flow rate, 1.4 mL/min; injector temperature, 120 °C; detector temperature, 250 °C; split ratio, 1:50.

The retention times of standards were as follows: rhamnose, 8.50 min; galactose, 11.99 min; α-D-glucose, 12.41 min; β-D-glucose, 13.78 min. The GC analysis of the TMS derivatives of $\mathbf{F_1}$ hydrolysates showed that $\mathbf{F_1}$ contained D-glucose.

Enzymatic Hydrolysis of F_4 by Tannase. The ^1H and ^{13}C NMR data of compound $\mathbf{F_4}$ revealed the presence of the ester moiety. To determine the type of ester group that was linked to the skeleton, $\mathbf{F_4}$ was hydrolyzed with tannase according to the method of Nonaka et al. (1983). Briefly, tannase (6 mg) was added to an aqueous solution of $\mathbf{F_4}$ (6 mg), and the mixture was shaken at room temperature for 1 h. This solution was evaporated to dryness at 40 °C under reduced

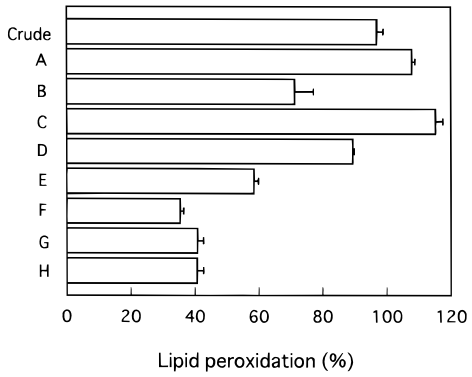

Figure 2. Rate of lipid peroxidation in the presence of the fractions (4.2×10^{-3}% w/v) separated by Sephadex LH-20 column chromatography. Data are mean ± SE of three experiments. The crude extract was used as a reference.

pressure, and methanol was added. The supernatant was used for the isolation of hydrolysates by preparative HPLC under the previously described conditions.

Peroxyl Radical-Scavenging Activity. The peroxyl radical-scavenging activity of each sample of the purification step (final concentration, 4.2×10^{-3}% w/v, ethanol solution) was determined as previously described (Watanabe et al., 1997) on the basis of the inhibition of the AMVN-initiated oxidation of methyl linoleate in solution. Each sample (50 μg) was added to the solution of methyl linoleate (0.1 M, 1.0 mL; hexane/2-propanol, 8:3 v/v) and preincubated at 37 °C for 5 min. The reaction was started by adding 0.1 M AMVN in *n*-hexane/2-propanol solution (8:3 v/v, 0.1 mL). The reaction mixture was incubated with continuous shaking at 37 °C in the dark. At 45-min intervals, aliquots of the mixture (20 μL) were injected into the HPLC column for the determination of the reaction products, methyl linoleate hydroperoxides (MeL-OOHs). The conditions of the HPLC were as follows: column, SIL (Cosmosil SIL, Nacalai Tesque Inc., Ltd.; 4.6 × 150 mm i.d.); mobile phase, 1.0% 2-propanol in hexane; flow rate, 2 mL/min; temperature, ambient; detection, UV detector (166 type, Beckman Co., Ltd.) (235 nm). The MeL-OOHs prepared as previously described were used for the calibration (Watanabe et al., 1995). The antioxidant activity of the fractions separated by Sephadex LH-20 was expressed as lipid peroxidation, calculated as the ratio of the rate of the production of MeL-OOHs in the presence of each fraction to the rate without samples, viz. control, during 90 min from the initiation of the experiment. A low lipid peroxidation rate in the presence of the samples means that their peroxyl radical-scavenging activity is high.

RESULTS

Antioxidant Activity and Contents of Fractions Separated by Sephadex LH-20. Figure 2 shows the antioxidant activity of the crude extract and seven fractions separated by Sephadex LH-20 column chromatography. Antioxidant activity was present in fractions B and D−H. The activity of all the active fractions was higher than that of the crude extract and was in the order F > G ≈ H > E > B > D. In contrast, fractions A and C revealed prooxidant activity.

Figure 1 shows the yields of these fractions. The yields of the active fractions D−H were lower than those of fractions A−C. The TLC chromatogram of fractions A and B is shown in Figure 3. The R_f values of the spots of fractions A and B, except for unknown immobilized ones, were 0.16 and 0.04, respectively, and they were compared with those in the literature (Snyder, 1973). Consequently, the antioxidant fraction B contained phospholipids, which have been reported as antioxidants (King et al., 1992; Ohshima et al., 1993) and synergists

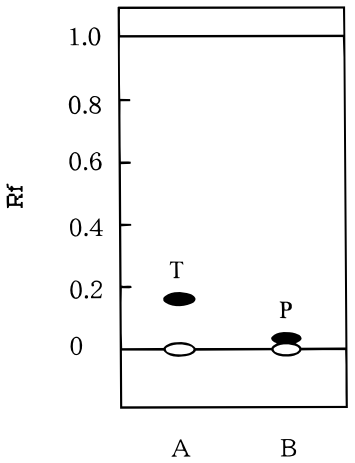

Figure 3. TLC chromatogram of fractions A and B for lipids. T, triacylglycerol; P, phospholipid.

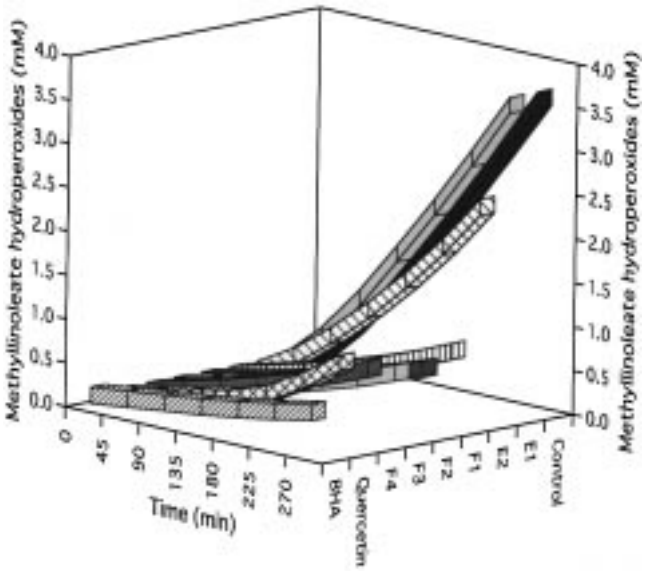

Figure 4. Effect of the isolated compounds (4.2×10^{-3}% w/v) from buckwheat groats on AMVN-initiated oxidation of methyl linoleate in solution. BHA and quercetin were used as reference standards.

with tocopherol (Lambelet et al., 1994), and the prooxidant fraction A contained triacylglycerols. Characterization of the prooxidant fraction C, the most major fraction, was still unclear.

Antioxidant Activity of the Isolated Compounds. Figure 4 shows the antioxidant activity of the isolated compounds. All of the isolated compounds, except **E_1**, exhibited antioxidant activity, lower than that of BHA. The activity of all compounds derived from fraction F, **F_1**−**F_4**, was higher than that of compound **E_2**. Among these compounds, the antioxidant activity was in the order **$F_2 \approx F_3 > F_4 \approx F_1$**, but the differences in activity were smaller than those between **E_2** and **F_1**−**F_4**. On the other hand, the activity of quercetin was slightly higher than that of compounds **F_1**−**F_4** during 180 min from the initiation of the lipid peroxidation, but it decreased rapidly thereafter, resulting in greater increases in the amount of the methyllinoleate hydroperoxides than compounds **F_1**−**F_4**.

Identification of the Isolated Antioxidant Compounds. *Identification of Compound **E_2**.* Compound **E_2** was identified as rutin on the basis of the spectral data of HPLC and ^{13}C NMR. The data were as follows:

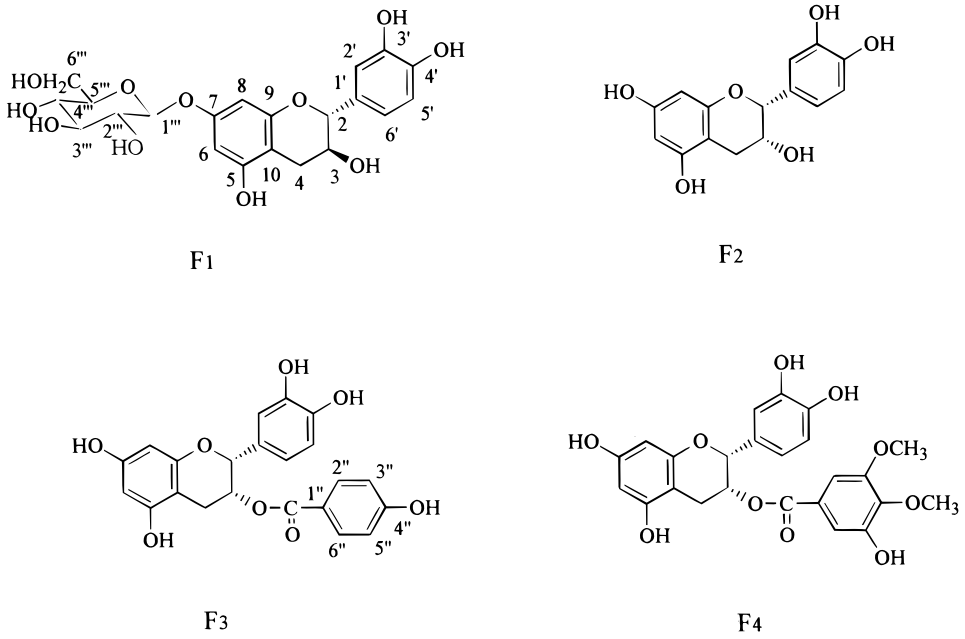

Figure 5. Structures of the isolated antioxidant compounds from buckwheat groats.

Table 1. ^{1}H NMR Spectral Data for Compounds F_1−F_4 (*J* in Hertz)

	F_1[a]	F_2[b]	F_3[b]	F_4[b]
H-2	4.77 d (7)	4.74 s	5.05 s	5.08 s
H-3	4.21 m	3.22 m	5.36 m	5.37 m
H-4	2.73 dd (8, 16)	2.46−2.71 m	2.67−2.98 m	2.72−3.00 m
	3.15 dd (6, 16)			
H-6	6.46 d (2)	5.89 d (2)	5.92 d (2)	5.85 d (2)
H-8	6.19 d (2)	5.73 d (2)	5.84 d (2)	5.94 d (2)
H-2′	7.03 d (2)	6.68 d (8)	6.90 s	6.91 s
H-5′	6.98 d (8)	6.89 d (8)	6.64 d (8)	6.67 d (8)
H-6′	6.90 dd (2, 8)	6.68 d (8)	6.71 d (8)	6.75 d (8)
H-2″			7.64 d (9)	6.87 d (2)
H-3″			6.78 d (9)	
H-5″			6.78 d (9)	
H-6″			7.64 d (9)	6.99 d (2)
OCH_3				3.70 s
				3.75 s

[a] Measured in acetone-d_6 + D_2O. [b] Measured in DMSO-d_6.

Table 2. ^{1}H NMR Spectral Data for Authentic Standard Catechins (*J* in Hertz)

	(+)-catechin[a]	(−)-epicatechin[b]	(−)-epicatechin gallate[b]
H-2	4.54 d (7)	4.74 s	5.03 s
H-3	3.99 m	3.22 m	5.36 m
H-4	2.44 dd (8, 16)	2.46−2.71 m	2.66−2.97 m
	2.76 dd (6, 16)		
H-6	5.95 d (2)	5.89 d (2)	5.94 d (2)
H-8	5.82 d (2)	5.73 d (2)	5.84 d (2)
H-2′	6.81 d (2)	6.68 d (8)	6.86 s
H-5′	6.75 d (8)	6.89 d (8)	6.65 d (8)
H-6′	6.68 dd (2, 8)	6.68 d (8)	6.75 d (8)
H-2″			6.82 s
H-6″			6.82 s

[a] Measured in acetone-d_6 + D_2O. [b] Measured in DMSO-d_6.

HPLC retention time 21.3 min; λ_{max} 258 and 356 nm; λ(shoulder) 266 and 296 nm; ^{13}C NMR (DMSO-d_6); δ 17.6 (rha CH_3), 66.9 (glu Cb), 68.1 (rha C5), 69.9 (glu C4), 70.3 (rha C3), 70.5 (rha C2), 71.8 (rha C4), 74.0 (glu C2), 75.8 (glu C5), 76.4 (glu C3), 93.5 (C8), 98.6 (C6), 100.6 (rha C1), 101.1 (glu C1), 103.8 (C10), 115.1 (C2′), 116.2 (C5′), 121.1 (C1′), 121.5 (C6′), 133.2 (C3), 144.6 (C3′), 148.3 (C4′), 156.3 (C2), 156.5 (C9), 161.1 (C5), 164.1 (C7), 177.2 (C4). All of these data were identical with those of rutin already isolated from buckwheat hulls.

Identification of Compounds ***F*****$_1$**−**F_4**. *(a) Compound* ***F*****$_2$**. This compound was identified as (−)-epicatechin (Figure 5) on the basis of the spectral data of ^{1}H (Table 1) and ^{13}C NMR (Table 3). All of these data were identical with that of the authentic standard (Tables 2, 4). The identity of this compound was further confirmed by cochromatography using HPLC.

(b) Compound ***F*****$_1$**. The data of ^{1}H and ^{13}C NMR suggested that this compound had (+)-catechin structure and a sugar moiety, which was identified as *O*-glucoside by trimethylsilylation. The carbon resonances of the glucose moiety were assigned on the basis of the comparison with the data in the literature (Markham and Chari, 1982), and the proton resonances were assigned by the ^{13}C−^{1}H COSY experiment. Moreover, the position of the glycosylated carbon of (+)-catechin was confirmed by 2D COLOC experiment (Figure 6). Detection of the long-range coupling between C-7 of the (+)-catechin skeleton at δ 156.2 (δ 156.4 in acetone-d_6 + D_2O) and the C1 anomeric proton of the sugar moiety at δ 4.72 indicated that D-glucose was linked to the C-7 position of the skeleton. The configuration of the glucose was determined to be β on the basis of the *J* value (7 Hz). In addition, a molecular ion at *m*/*z* 453 $[M + H]^+$ was detected by FAB-MS analysis. From these data, this compound was identified as (+)-catechin 7-*O*-β-D-glucopyranoside (Figure 5).

(c) Compound ***F*****$_3$**. The data of ^{1}H and ^{13}C NMR suggested the presence of (−)-epicatechin structure in this compound. A_2B_2-type aromatic signals found at δ 5.84 and 5.92 (each 2H, d, J = 8 Hz) in ^{1}H NMR suggested the presence of the *p*-hydroxyphenyl ring. The ^{13}C NMR data also supported the esterification of the (−)-epicatechin skeleton by this group. The location of the ester moiety was established by ^{13}C NMR data by the lowfield shift of C-3 (3.3 ppm) and the highfield shift of C-2 (1.2 ppm) and C-4 (2.1 ppm) of the skeleton, and the change in chemical shifts was similar to that of (−)-epicatechin 3-*O*-gallate compared with (−)-epicatechin. In addition, a molecular ion at *m*/*z* 411 $[M + H]^+$ was

Table 3. ^{13}C NMR Spectral Data for Compounds F_1−F_4 Measured in Acetone-d_6 + D_2O

	F_1	F_2	F_3	F_4
C2	81.6	78.6	77.4	77.3
C3	67.2	66.2	69.5	70.3
C4	27.5	28.0	25.9	25.7
C5	156.7	156.5	156.5	156.6
C6	97.5	96.0	96.1	96.1
C7	156.5	156.2	156.5	156.6
C8	96.3	95.4	95.2	95.2
C9	155.5	156.2	156.1	156.1
C10	102.8	99.7	98.5	98.4
C1′	130.9	131.3	130.5	130.5
C2′	115.1	115.7	114.5	114.3
C3′	145.0	144.4	144.6	144.8
C4′	144.8	144.5	144.7	144.7
C5′	116.0	114.8	115.6	115.7
C6′	119.7	119.0	118.6	118.4
−COO−			166.6	166.3
C1″			121.2	125.6
C2″			132.1	105.6
C3″			115.7	153.3
C4″			162.0	141.2
C5″			115.7	150.3
C6″			132.1	111.1
OCH_3				56.2
				60.7
C1‴	101.1			
C2‴	73.4			
C3‴	76.4			
C4‴	70.1			
C5‴	76.7			
C6‴	61.3			

Table 4. ^{13}C NMR Spectral Data for Authentic Standard Catechins Measured in Acetone-d_6 + D_2O

	(+)-catechin	(−)-epicatechin	(−)-epicatechin gallate
C2	81.5	78.6	77.5
C3	67.3	66.2	69.7
C4	27.6	28.0	25.9
C5	156.2	156.5	156.6
C6	95.9	96.0	96.1
C7	156.4	156.2	156.5
C8	95.1	95.4	95.3
C9	155.8	156.2	156.2
C10	100.3	99.7	98.5
C1′	131.1	131.3	130.5
C2′	115.0	115.7	114.6
C3′	144.8	144.4	144.7
C4′	144.9	144.5	144.7
C5′	116.0	114.8	115.7
C6′	119.7	119.0	118.8
−COO−			166.8
C1″			120.4
C2″			109.7
C3″			145.3
C4″			141.2
C5″			145.3
C6″			109.7

detected by FAB-MS analysis. From these data, this compound was identified as (−)-epicatechin 3-*O*-*p*-hydroxybenzoate (Figure 5).

(d) Compound ***F*****$_4$**. The data of ^{1}H and ^{13}C NMR suggested that this compound had (−)-epicatechin structure. The ^{1}H NMR data showed the presence of the two methoxyl groups (OMe), and the chemical shift of ^{13}C NMR (δ 56.2 and 60.7) showed that the OMe was diortho-substituted by another OMe and OH (Panichpol and Waterman, 1978). Furthermore, the ^{13}C NMR data showed the presence of the ester moiety (δ 166.3). To confirm the moiety, two enzymatic hydrolysates of compound **F$_4$**, **F$_4$-a** and **F$_4$-b**, recovered by preparative HPLC, were analyzed by ^{1}H and ^{13}C NMR. The NMR

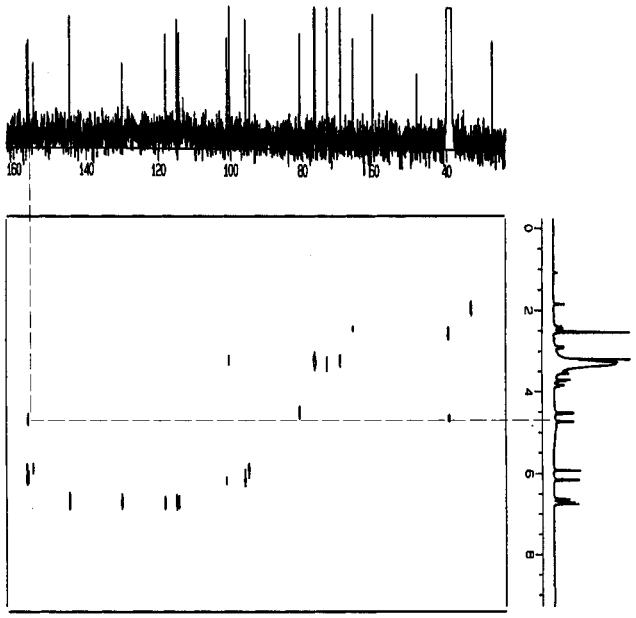

Figure 6. Heteronuclear long-range correlations observed by COLOC experiment in compound **F$_1$**. DMSO-d_6 was used as the solvent.

data of compound **F$_4$-a** were consistent with those of **F$_2$**; hence, this compound was identified as (−)-epicatechin. The data on ^{1}H and ^{13}C NMR of F_4-b were as follows: ^{1}H NMR (DMSO-d_6) δ 3.71 (s, OCH_3), 3.78 (s, OCH_3), 7.03 (d, J = 2 Hz), 7.09 (d, J = 2 Hz); ^{13}C NMR (DMSO-d_6) δ 55.6 (OCH_3), 61.0 (OCH_3), 104.5 (C2), 110.7 (C6), 124.5 (C1), 139.4 (C4), 149.9 (C5), 152.4 (C3), 167.5 (C=O). The meta-coupled doublets of aromatic protons at δ 7.03 and 7.09 in the ^{1}H NMR data of **F$_4$-b** suggested that this compound had the unsymmetrical 3,4,5-trisubstitution pattern. In addition, two OMe groups found in intact compound **F$_4$** were also detected in **F$_4$-b** (δ 3.71 and 3.78), the positions of which were determined as C-3 and C-4 by the cross-peaks of the 2D COLOC experiment between each carbon and the protons of OMe, which were clearly observed in compound **F$_4$** (Figure 7). Furthermore, a carboxyl carbon was found in the ^{13}C NMR spectrum (δ 167.5). From these data, **F$_4$-b** was identified as 3,4-di-*O*-methylgallic acid. The position of esterification of **F$_4$-a** by **F$_4$-b** was deduced from the change in the chemical shifts of ^{13}C NMR data of compound **F$_4$** compared with (−)-epicatechin. The C-3 of the carbon signal (δ 70.8) was deshielded by 4.1 ppm, and the C-2 (δ 77.3) and C-4 (δ 25.7) were shielded by 1.3 and 2.3 ppm, respectively. These results indicated that the ester moiety was located at the C-3 position of the (−)-epicatechin skeleton. In addition, a molecular ion at m/z 471 $[M + H]^+$ was detected by FAB-MS analysis. From these data, this compound was established as (−)-epicatechin 3-*O*-(3,4-di-*O*-methyl)gallate (Figure 5).

(e) Detection of (+)-Catechin. Along with the isolation of these antioxidant compounds, (+)-catechin was detected as a minor component in fraction F and was confirmed with the HPLC spectral data in comparison with an authentic standard and cochromatography. The retention time of (+)-catechin was 11.0 min under the condition of 5−95% methanol over 30 min.

DISCUSSION

In this experiment, we obtained four catechins along with rutin with peroxyl radical-scavenging activity and,

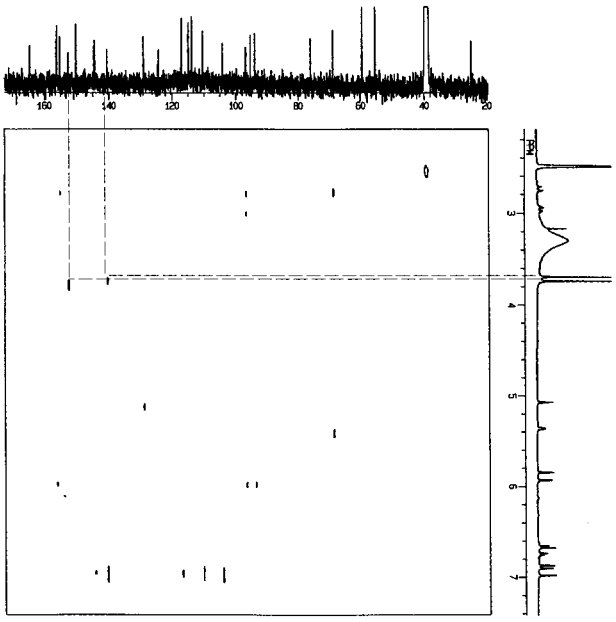

Figure 7. Heteronuclear long-range correlations observed by COLOC experiment in compound $\mathbf{F_4}$. DMSO-d_6 was used as the solvent.

moreover, detected (+)-catechin. The yields of the isolated compounds suggest that the total amount of catechins contained is more than rutin, at least in this cultivar (Iwate zairai) of buckwheat groats. Besides, the presence of glycoside and ester derivatives of catechins as major components has not been reported for cereals or other crops, except for tea leaves. Catechins are abundant in green tea leaves, and many of their biological activities have been reported, such as antioxidant activity (Scott et al., 1993; Matsuzaki and Hara, 1985; Lunder, 1992), hypocholesterolemic effect (Muramatsu et al., 1986; Fukuyo et al., 1986), antitumor activity (Hara et al., 1989; Kada et al., 1985), antibacterial activity (Hara and Watanabe, 1989; Hara and Ishigami, 1989), and inhibitory activity of the angiotensin I converting enzyme (ACE) (Hara et al., 1987). The inhibitory activity of the ACE of many foods has already been investigated, and that of the buckwheat flour was found to be particularly strong (Suzuki et al., 1983). The active principle in the flour was presumed to be a heat stable and low molecular weight substance (Suzuki et al., 1983). Subsequently, a tripeptide was isolated from buckwheat seeds (Suzuki, 1989). Although the inhibitory activity of ACE gradually increased from the outer layer toward the inner layer within the buckwheat seed (Suzuki et al., 1987), the contents of many components, including nutritive ones (protein, lipid, and minerals) in the outer layer of the groat were higher than those in its inner layer (Kusano and Miyashita, 1973; Kusano et al., 1974). Hence, the localizations of the peptide and catechins in buckwheat groats should be investigated for the elucidation of mainly contributory components to the ACE inhibitory activity.

The antioxidant activity of the isolated compounds showed that the activity of catechins was superior to that of rutin, which is known as an antioxidant in buckwheat, at the same concentration. Bors et al. (1990) showed that the strong radical-scavenging activity of quercetin depends on its structures such as 2,3-double bond conjugation with a 4-oxo function and the presence of both 3- and 5-hydroxyl groups along with catechol structure in the B-ring (Bors et al., 1990). Catechins have 2,3-saturated bond and no 4-oxo function, resulting in no electron delocalization between the A and B rings (Rice-Evans et al., 1996). Although the initial peroxyl radical-scavenging activities of catechins in the experiment, derived from their catechol structure, were lower than that of quercetin, their functional time as scavengers should be longer than that of quercetin. For isolated catechins, if the substituted groups of the (−)-epicatechin or (+)-catechin skeletons have no effect on the peroxyl radical scavenging activity, the activity of $\mathbf{F_2}$ should be highest at the same concentration (w/v). The equivalent antioxidant activity of compound $\mathbf{F_3}$ (MW 410) and compound $\mathbf{F_2}$ (MW 290) indicates that the esterification of the skeleton by the hydroxylated phenolic group at the C-3 position would contribute to the activity. Further study is required for the elucidation of the relationship between the high peroxyl radical-scavenging activity and the structure of catechins.

Flavonoids are synthesized from a molecule of phenylalanine derived from the shikimate pathway and three molecules of acetic acid. The total content of the flavonoids in buckwheat groats may be regulated by the key enzymes of the initial steps of flavonoid biosynthesis, such as phenylalanine ammonia-lyase (PAL) and chalchone synthase. Catechins are synthesized by the double-step reaction of NADPH-dependent reductases from 3-OH flavanone via leucoanthocyanidin (Stafford and Lester, 1985). On the other hand, flavonols are supposed to be synthesized also from 3-OH flavanone by a dioxygenase type of flavonol synthase (Stafford, 1991). In this variety, relatively high yields of glycoside ($\mathbf{F_1}$) and methylated catechin ($\mathbf{F_4}$) show that glycosyltransferase and methyltransferase would be highly active in the final step of the biosynthesis. The contents of rutin and catechins among varieties of buckwheat groats may be attributed to the mass or the activity of either or both of these enzymes in the synthetic parts of these flavonoids during the ripening period. We are now measuring the content of the isolated antioxidant compounds in the groats of 60 buckwheat varieties harvested this year.

The identification of the antioxidant compounds from other fractions, G and H, with high antioxidant activity, has been superficial. The results of the TLC chromatogram of these fractions revealed the presence of compounds that possess flavan-3-ol skeletons. The isolation and identification of the antioxidant compounds from these fractions are in progress.

ABBREVIATIONS USED

AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); HPLC, high-performance liquid chromatography; MeL-OOH, methyl linoleate hydroperoxide; TLC, thin-layer chromatography; FAB-MS, fast atom bombardment mass spectrometry; NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; COLOC, correlation spectroscopy via long-range coupling.

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