Antioxidant Compounds from Buckwheat (*Fagopyrum esculentum* Möench) Hulls

Mitsuru Watanabe,*,† Yasuo Ohshita,†,‡ and Tojiro Tsushida§

Tohoku National Agricultural Experiment Station, Ministry of Agriculture, Forestry and Fisheries, Morioka, Iwate 020-01, Japan, and National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki 305, Japan

Ethanolic extracts of buckwheat (*Fagopyrum esculentum* Möench) hulls were separated by Sephadex LH-20 column chromatography into eight fractions. Five of the fractions exhibited peroxyl radical-scavenging activity by inhibiting the oxidation of methyl linoleate in solution. Two of the antioxidant fractions contained proanthocyanidins (condensed tannins) from the color reaction of these fractions with HCl under heat treatment. Five antioxidant compounds were isolated by preparative HPLC and identified as quercetin, hyperin, rutin, protocatechuic acid, and 3,4-dihydroxybenzaldehyde. The contents of these active compounds in the buckwheat hulls were as follows: protocatechuic acid (13.4 mg/100 g of dried hulls), 3,4-dihydroxybenzaldehyde (6.1 mg/100 g), hyperin (5.0 mg/100 g), rutin (4.3 mg/100 g), and quercetin (2.5 mg/100 g). Besides the isolation of these compounds, two major compounds that showed no peroxyl radical-scavenging activity in the extract were isolated and identified as vitexin and isovitexin.

Keywords: *Fagopyrum esculentum; antioxidant activity; flavonoids; phenolic compounds; proanthocyanidin*

INTRODUCTION

The main role of the seed is to leave its offspring safe from environmental stresses (dryness, low temperature, etc.). The seed coat, which covers the seed, plays the major role in the physical and chemical defense systems of the seed. Antioxidant compounds in plants, for example, tocopherols, carotenoids, and other phenolic compounds, are effective in the protection against oxidative damage toward membranes that contain polyunsaturated fatty acids (Osawa et al., 1985). Therefore, many plants were investigated as sources of natural antioxidants; a great variety of compounds have been isolated, many of which are phenolic compounds (Nakatani, 1990; Osawa et al., 1992).

For the phenolic compounds in buckwheat, flavonols such as rutin, hyperin, quercitrin, and quercetin were isolated from its immature seeds (Sato and Sakamura, 1975), and flavones such as vitexin, isovitexin, orientin, and isoorientin were detected in its seedlings (Margna and Margna, 1982). On the other hand, Durkee et al. (1977) reported on the occurrence of syringic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, and proanthocyanidins in the bran-aleurone layer of the buckwheat seed. However, the study of antioxidant activity of phenolic compounds in this crop thus far has been superficial. In our previous paper, we have reported that the ethanolic extract from buckwheat seeds had a higher antioxidant activity than the extract using other solvents (petroleum ether and ethyl acetate), and the activity of ethanolic extracts varied substantially among the varieties (Watanabe et al., 1995). In this paper, we describe the isolation and identification of antioxidant compounds from buckwheat hulls.

MATERIALS AND METHODS

Materials. Buckwheat (*Fagopyrum esculentum* Möench. cv. Iwate zairai) was grown at the Tohoku National Agricultural Experiment Station in 1993. After the harvest, the seeds were dried at ambient temperature with ventilation and dehulled by a grinder with care to prevent crushing of the groats. The samples were divided into hulls and groats by gently blowing the hulls off using an electric fan.

Chemicals. All of the authentic flavonoids (quercetin, rutin, hyperin, vitexin, and isovitexin) and anthocyanidins (cyanidin and pelargonidin) were obtained from Extrasynthèse S.A. (Genay, France). Protocatechuic acid, 3,4-dihydroxybenzaldehyde, and methyl linoleate were purchased from Tokyo Kasei Organic Chemicals Co., Ltd. (Tokyo, Japan), and the methyl linoleate was purified by column chromatography as described previously (Terao et al., 1986). 2,2′-Azobis(2,4-dimethylvaleronitrile) was obtained from Wako Pure Chemicals Industries (Osaka, Japan). BHA was obtained from Nacalai Tesque Inc., Ltd. (Kyoto, Japan).

Extraction. Figure 1 shows the scheme for the preparation of antioxidant compounds from buckwheat hulls. Separated hulls from buckwheat seeds were dried at 60 °C and ground. The powdered sample (300 g) was extracted with ethanol under reflux in a water bath at 80 °C for 1 h, and the solid was removed by filtration using Advantec No. 5C (Toyo Inc.) filter paper. The collected extract was evaporated under reduced pressure at 40 °C, and the condensed ethanolic solution was used as the crude extract.

Isolation of Antioxidant Compounds from the Ethanolic Extract of Buckwheat Hulls. *(a) Column Chromatography.* The crude extract was applied to the Sephadex LH-20 (Pharmacia Co., Ltd.) column (46 × 805 mm i.d.) and eluted stepwise with ethanol, methanol, and 70% acetone with monitoring at 280 and 350 nm. The ethanolic eluate was divided into six fractions (Figure 2). Each of the eight fractions obtained was concentrated under reduced pressure at 40 °C. Antioxidant activity was found in fractions D−H.

(b) Preparative HPLC. Except for fractions G and H, whose HPLC chromatograms showed minor peaks that were different from major ones found in the chromatogram of the crude

* Author to whom correspondence should be addressed (telephone -81-19-643-3513; fax -81-19-641-7794).

† Tohoku National Agricultural Experiment Station.

‡ Present address: Hokkaido National Agricultural Experiment Station.

§ National Food Research Institute.

S0021-8561(96)00555-9 CCC: $14.00

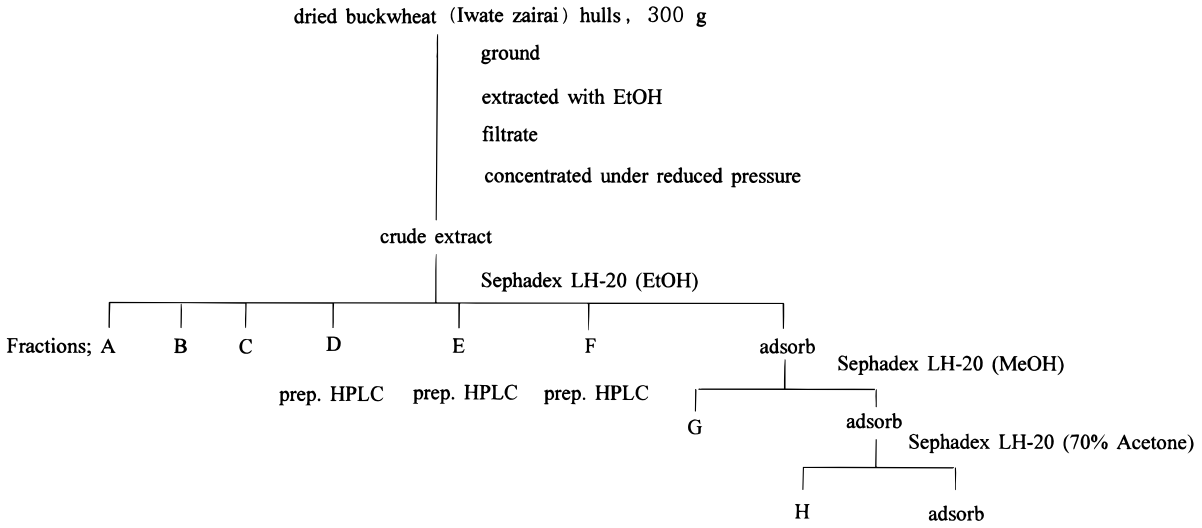

Figure 1. Scheme for preparation of antioxidant fractions and compounds from buckwheat hulls.

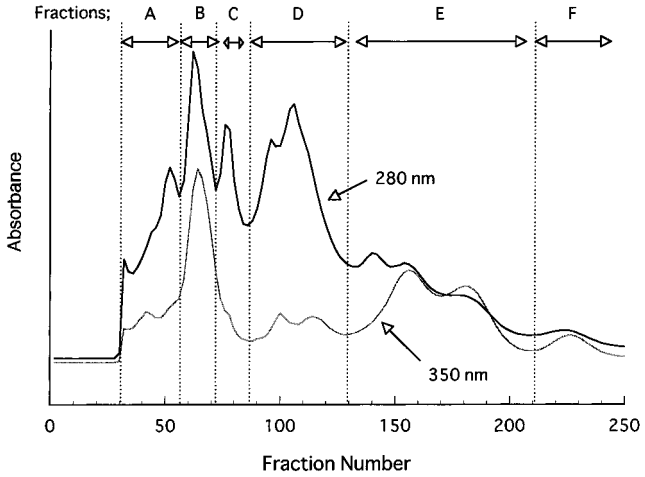

Figure 2. Chromatogram of the crude extract by Sephadex LH-20 column chromatography eluted with ethanol. Eluate of initial 320 mL was discarded. Fractions of 5 mL were collected.

extract, main peaks in active fractions D−F were isolated by HPLC fitted with an ODS column with repeated injections. 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); 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 and checked for purity by TLC and HPLC.

Identification and Structure Analysis of the Isolated Compounds. The identification of flavonols already found in buckwheat seeds (Sato and Sakamura, 1975) was based on the comparison of chromatographic and spectral data between the isolated compounds and standards. For other isolated compounds, instrumental analysis (FAB-MS and ^{1}H NMR) was performed. Moreover, all of them were identified with co-chromatography using HPLC and TLC.

(a) Analytical HPLC and TLC. To check the purity of the isolated compounds and to identify them, these compounds were analyzed by HPLC and TLC.

The conditions of the HPLC were as follows: column, ODS (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; flow rate, 1 mL/min; detection, photodiode array detector (168 type, Beckman Co., Ltd.; on-line acquisition of absorption spectra, 240−400 nm).

The conditions of TLC were as follows: TLC plates (cellulose; 2−15 μm particle size, 0.1 mm thickness; Merck). First, chromatograms were developed in BAW (*n*-butanol−acetic acid−water, 4:1:5 v/v/v), and, second, they were developed in 30% acetic acid. The chromatograms were made visible by UV, UV with ammonia vapor, and UV with spraying an $AlCl_3$ reagent.

(b) Fast Atom Bombardment Mass Spectrometry (FAB-MS). FAB-MS spectra of the isolated compounds were recorded on a JEOL JMS-SX102A mass spectrometer with glycerol as the mounting matrix.

(c) ^{1}H NMR. ^{1}H NMR (400 MHz) spectra of the isolated compounds were recorded on a JEOL JNM-EX 400 spectrometer with CD_3OD as the solvent. To determine the chemical shifts given in the ∂ value (ppm), the peak of the solvent (∂ 3.3 ppm) was used as the reference peak.

Hydrolysis of Fractions G and H. The tailing spots by TLC chromatograms of fractions G and H under condition as previously described with spraying vanillin-HCl reagent (Ribereau-Gayon, 1972) suggested that proanthocyanidins (condensed tannins) were contained in these fractions. To confirm the presence of proanthocyanidins, these fractions were hydrolyzed by the method of Porter et al. (1986) in the following manner: To 1 mL of the methanolic solutions of these fractions was added 6 mL of BuOH−concentrated HCl (95:5 v/v) and 0.2 mL of a 2% solution of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 2 M HCl. The mixed solutions were heated at 95 °C in a water bath for 2 h and then cooled. From the result of this experiment, that is, the solution turning red, it was shown that these two fractions contained proanthocyanidin. Proanthocyanidins are polymers of flavan monomer extender units (Figure 3), and they cover a wide range of molecular weights. Consequently, these two fractions were used for the determination of the type of extender units by TLC analysis, and the acetyl derivatives of these fractions were used for the determination of the degree of polymerization of these units by gel permeation chromatography (GPC).

TLC Analysis of Hydrolysates of Fractions G and H. The hydrolysates of fractions G and H were analyzed by TLC (cellulose plate) under the following conditions: first, they were developed in a Forestal solvent (acetic acid−HCl−H_2O, 30:3:10 v/v/v), and, second, they were developed in BAW (4:1:5 v/v/v).

Acetylation of Fractions G and H. The methanolic solution of fractions G and H was acetylated in the following way: To 1 mL of each solution was added 1 mL of pyridine and 1 mL of anhydrous acetic acid; the solutions were then left overnight at room temperature. H_2O and ethyl acetate were added to the mixture. The ethyl acetate layer was washed with H_2O and evaporated to dryness under reduced pressure at 40 °C.

GPC Analysis of Acetyl Derivatives of Fractions G and H. Acetyl derivatives of fractions G and H were dissolved in THF and submitted for GPC analysis to investigate their factors with respect to average molecular weight with a

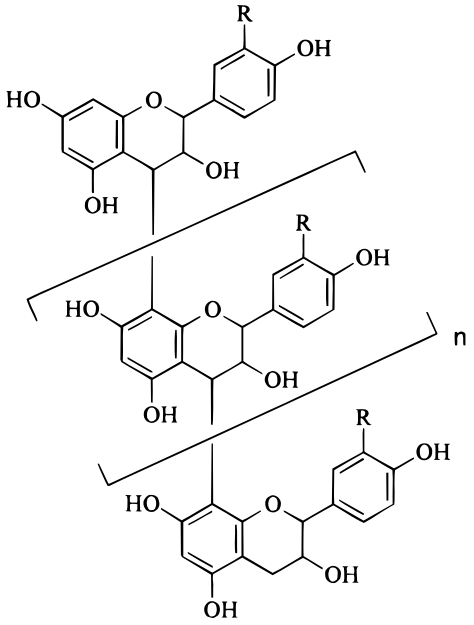

Figure 3. Proanthocyanidin structure. R = OH, procyanidin (extender unit = catechin and/or epicatechin). R = H, propelargonidin (extender unit = afzelechin and/or epiafzelechin).

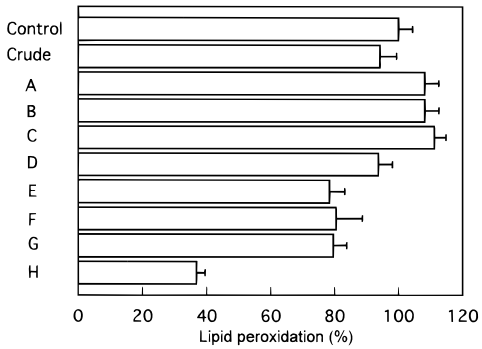

Figure 4. Rate of lipid peroxidation in the presence of the fractions (4.2×10^{-3}% wt/v) separated by Sephadex LH-20 column chromatography. Data are mean ± SD of two experiments except for control ($n = 3$). The crude extract was used as a reference.

calibration curve using standard polystyrenes (Easi Cal, (PL Laboratory Co., Ltd.). The conditions of GPC analysis were as follows: column, PLgel 5μ MIXD-C (7.5 × 600 mm i.d.) (PL Laboratory Co., Ltd.); mobile phase, THF; flow rate, 1 mL/min; detection, refractive index (RI) detector (410 type, Waters Co., Ltd.). The following factors were investigated: (1) number-average molecular weight (M_n), (2) weight-average molecular weight (M_w), and (3) M_w/M_n.

Peroxyl Radical-Scavenging Activity. The peroxyl radical-scavenging activity of 50 μg of each of the crude extract, fractions separated by Sephadex LH-20, and the isolated compounds (final concentration, 4.2×10^{-3}% wt/v, ethanol or dimethylformamide−ethanol solution) was determined according to the method of Terao et al. (1993), based on the inhibition of the AMVN-initiated oxidation of methyl linoleate in solution. Each sample was added to the solution of methyl linoleate (0.1 M, 1.0 mL; hexane/isopropyl alcohol, 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/isopropyl alcohol solution (8:3 v/v, 0.1 mL). The reaction mixture was incubated with continuous shaking at 37 °C in the dark. At specific intervals, aliquots of the mixture (20 μL) were injected into the HPLC column for determination of the 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% isopropyl alcohol in hexane; flow rate, 2 mL/min; detection, UV detector (166 type, Beckman Co., Ltd.; 235 nm). The antioxidant activity of the fractions separated by Sephadex LH-20 was expressed as the ratio of the rate of the production of MeL-OOHs in the presence of each fraction to the rate without samples, "control," during 90 min from the initiation of the experiment. A low ratio in the presence of the samples means that their peroxyl radical-scavenging activity is high.

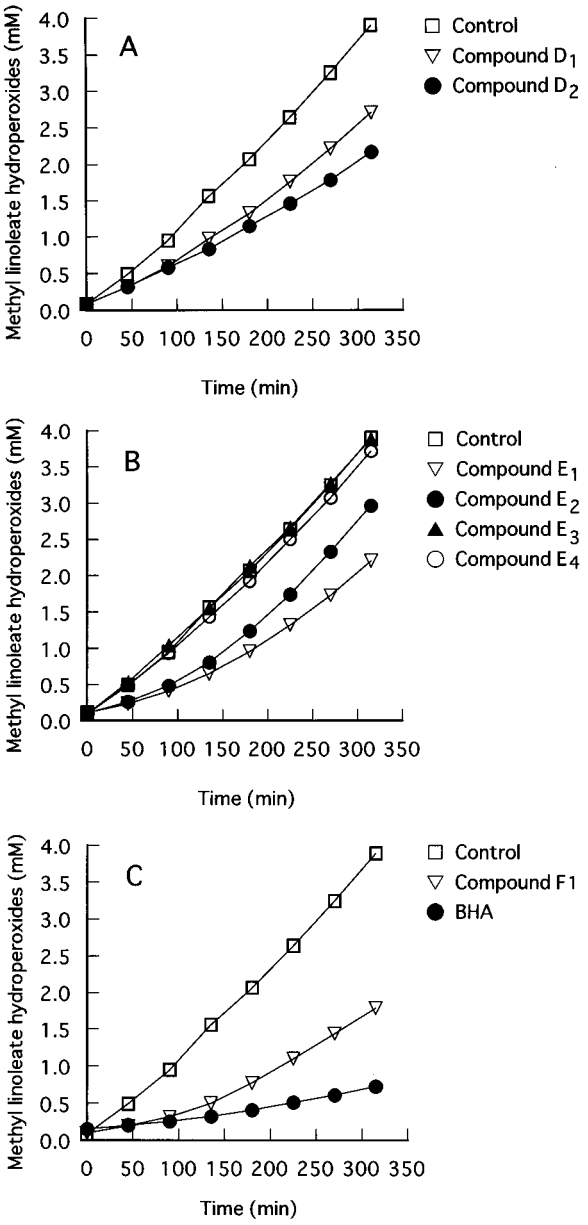

Figure 5. Effect of the isolated compounds (4.2×10^{-3}% wt/v) from buckwheat hulls on AMVN-initiated oxidation of methyl linoleate in solution. BHA was used as a reference standard. (A) Compounds from fraction D. (B) Compounds from fraction E. (C) Compound from fraction F and BHA.

RESULTS

Peroxyl Radical-Scavenging Activity of Fractions Separated by Sephadex LH-20. The peroxyl radical-scavenging activity of the fractions separated by Sephadex LH-20 is shown in Figure 4. The activity of fractions E−H was higher than that of the crude extract, and the activity of fraction D was almost the same as that of the crude extract. However, fractions A−C, which were also major constituents in the ethanolic extract (Figure 2) from buckwheat hulls, exhibited no activity.

Table 1. Retention Times and UV Spectral Data of the Isolated Compounds from Buckwheat (*Iwate zairai*) Hulls by Photodiode Array Detector

compound	retention time (min)	λ_{max} (nm)	λ(shoulder) (nm)
E_1	20.7	257, 355	267, 296
E_2	21.3	258, 356	266, 296
E_3	16.4	270, 339	300
E_4	19.2	271, 339	
F_1	35.7	256, 370	272, 301
D_1	5.6	282, 312	
D_2	4.6	262, 296	

Peroxyl Radical-Scavenging Activity of the Isolated Compounds. Seven compounds were isolated from fractions D−F: compounds D_1 (9.5 mg) and D_2 (6.3 mg) from fraction D; compounds E_1 (2.5 mg), E_2 (2.2 mg), E_3 (6.1 mg), and E_4 (8.4 mg) from fraction E; and compound F_1 (2.2 mg) from fraction F. Figure 5 shows the peroxyl radical-scavenging activity of the isolated compounds. Although compounds D_1, D_2, E_1, E_2, and F_1 showed peroxyl radical-scavenging activity, their activity was lower than that of BHA at the same concentration (4.2×10^{-3}% wt/v), and compounds E_3 and E_4 showed almost no activity. Of the isolated active compounds, peroxyl radical-scavenging activity during the first 90 min of the experiment was in the following order: compound F_1, E_1, E_2, D_2, and D_1, and thereafter the activity of compound E_2 rapidly decreased. Because the change in the rate of the production of MeL-OOHs in the presence of D_2 during the experiment was smaller than any other isolated compounds, this compound would act as an antioxidant for a long time at the same concentration.

Identification of the Isolated Compounds from Fractions D−F. *Compounds E_1−E_4 and F_1.* Table 1 shows the chromatographic and spectral data of the isolated compounds by HPLC. Compounds E_1−E_4 and F_1 exhibited two absorption maxima from 240 to 400 nm. The two major absorption peaks of flavones and flavonols in methanol are referred to as Bands I (usually 300−380 nm) and II (usually 240−280 nm), which are attributed to the B-ring cinnamoyl system and the A-ring benzoyl system, respectively (Mabry et al., 1970). These chromatographic and spectral data indicate that these isolated compounds are either flavonol or flavone. From the shape of the absorption spectra of compounds E_1−E_4 and F_1 compared with that in the literature (Markham, 1982), it was suggested that compounds E_1 and E_2 were 3-OH-substituted flavonols that contain quercetin as aglycon; compound F_1 was a 3-OH free flavonol, quercetin; compounds E_3 and E_4 were flavones that have apigenin as aglycon.

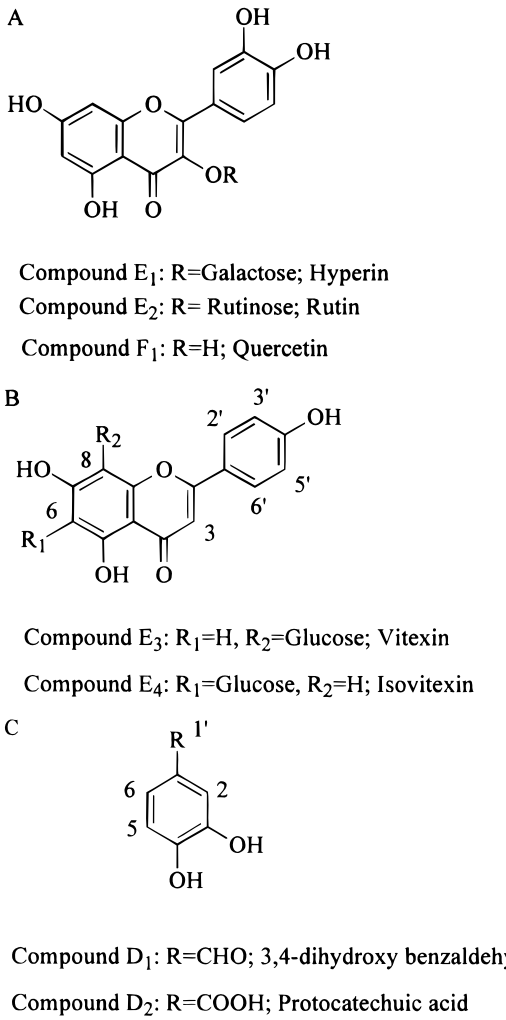

Figure 6. Structure of the isolated compounds from buckwheat hulls. (A) Flavonol. (B) C-glycosyl flavone. (C) Dihydroxy phenolic compounds.

The identity of compounds E_1, E_2, and F_1 was established by comparison of the chromatographic data with authentic flavonols and co-chromatography using HPLC and TLC (Table 2). Consequently, compounds E_1, E_2, and F_1 were identified as hyperin, rutin, and quercetin, respectively (Figure 6).

Compounds E_3 and E_4 were identified as vitexin and isovitexin, respectively. Vitexin and isovitexin are isomers, and they differ in the position of the C-linked glucose. The FAB-MS of compounds E_3 and E_4 gave a molecular ion at m/z 433 $[M+H]^+$. The ^1H NMR data of compounds E_3 and E_4 (Table 3) were consistent with

Table 2. Thin-Layer Chromatographic Data of the Isolated Compounds from Buckwheat (*Iwate zairai*) Hulls and Standard Flavonoids

	R_f value		color		
compound	BAW	30% AcOH	UV	UV/NH_3	UV/$AlCl_3$
E_1	0.69	0.47	dark purple	fluorescent yellow	fluorescent yellow
E_2	0.53	0.63	dark purple	fluorescent yellow	fluorescent yellow
E_3	0.45	0.29	dark purple	fluorescent yellow	fluorescent yellow
E_4	0.79	0.62	dark purple	fluorescent yellow	fluorescent yellow
F_1	0.68		yellow	fluorescent yellow	fluorescent green-yellow
D_1	0.90	0.71	fluorescent blue	fluorescent blue	fluorescent blue
D_2	0.86	0.65	fluorescent blue	fluorescent blue	fluorescent blue
hyperin	0.67	0.45	dark purple	fluorescent yellow	fluorescent yellow
rutin	0.52	0.67	dark purple	fluorescent yellow	fluorescent yellow
vitexin	0.50	0.30	dark purple	fluorescent yellow	fluorescent yellow
isovitexin	0.75	0.58	dark purple	fluorescent yellow	fluorescent yellow
quercetin	0.68		yellow	fluorescent yellow	fluorescent green-yellow

Table 3. ^{1}H NMR Data of the Isolated Compounds E_3, E_4, D_1, and D_2[a]

δ compound E_3	δ compound E_4	δ compound D_1	δ compound D_2
7.98 (2H, d, H2′, H6′, J = 8.8)	7.85 (2H, d, H2′, H6′, J = 8.8)	9.68 (1H, s, H1′)	7.42 (1H, s, H2)
6.94 (2H, d, H3′, H5′, J = 8.8)	6.93 (2H, d, H3′, H5′, J = 8.8)	7.30 (1H, d, H6, J = 7.2)	7.41 (1H, d, H6, J = 7.2)
6.60 (1H, s, H3)	6.62 (1H, s, H3)	7.29 (1H, s, H2)	6.78 (1H, d, H5, J = 7.2)
6.26 (1H, s, H6)	6.52 (1H, s, H8)	6.90 (1H, d, H5, J = 7.2)	
3.48−4.13 (6H, glucose)	3.47−4.15 (6H, glucose)		

[a] s, Singlet; d, doublet.

those of authentic vitexin; apigenin-8-C glucoside, and authentic isovitexin; apigenin-6-C glucoside, respectively (Figure 6). The signal of the glucose C-1 proton of these 6- and 8-C-glycosides, which were usually found downfield from the bulk of the sugar protons, in the region ∂ 4.8−5.2 of trimethylsilylated flavonoids in CCl_4 (Mabry et al., 1970), was overlapped with the signal of CD_3OH at ∂ 4.81.

Compound D_1. Compound D_1 was identified as 3,4-dihydroxybenzaldehyde (Figure 6). The FAB-MS of compound D_1 gave a molecular ion at m/z 139 $[M+H]^+$. The ^{1}H NMR data of this compound (Table 3) were identical with those of authentic 3,4-dihydroxybenzaldehyde.

Compound D_2. Compound D_2 was identified as protocatechuic acid (Figure 6). The FAB-MS of compound D_2 gave a molecular ion at m/z 155 $[M+H]^+$. The ^{1}H NMR data of this compound (Table 3) were identical with those of authentic protocatechuic acid.

Partial Characterization of Fractions G and H. Fractions G (365.1 mg) and H (15.1 mg) contained proanthocyanidin as previously described. By TLC analysis, the acid hydrolysates of fraction G contained cyanidin, pelargonidin, and a small amount of other constituents. In contrast, the acid hydrolysates of fraction H contained a small amount of procyanidin and other constituents. Furthermore, by the GPC analysis of these fractions, M_n, M_w, and M_w/M_n of the acetyl derivatives of fraction G were calculated as 1100, 7300, and 6.6, respectively, and those of fraction H were calculated as 373, 5650, and 15.1, respectively. From this result it was suggested that fraction G contains various degrees of polymerization of proanthocyanidins, and fraction H contains a large amount of low molecular weight compounds along with proanthocyanidins.

Contents of the Isolated Antioxidant Compounds in Buckwheat Hulls. To investigate the contribution of the isolated compounds to the peroxyl radical-scavenging activity of the crude extract, the contents of all of the isolated compounds in the buckwheat hulls were determined by HPLC analysis using an ethanolic extract. The contents were in the following order: protocatechuic acid (13.4 mg/100 g of dried hulls) > 3,4-dihydroxybenzaldehyde (6.1 mg/100 g) > hyperin (5.0 mg/100 g) > rutin (4.3 mg/100 g) > quercetin (2.5 mg/100 g). The contents of the isolated antioxidant compounds in the buckwheat hulls were equivalent to rutin which is rich in its plants and groats. In addition to this, the amounts of fractions G and H were 121.7 (mg/100 g) and 5.0 (mg/100 g), respectively. The contents of vitexin and isovitexin were 4.6 (mg/100 g) and 3.3 (mg/100 g), respectively.

DISCUSSION

In this experiment, we isolated five compounds, that is, quercetin, rutin, hyperin, protocatechuic acid, and 3,4-dihydroxybenzaldehyde, from buckwheat hulls with peroxyl radical-scavenging activity. The last one is a novel compound isolated from buckwheat, and it has been isolated as a fungistatic substance against *Gloeosporium musarum* from green cavendish bananas (Mulvena, 1969). Protocatechuic acid, the ethyl ester of which was used as an antioxidant of food additives, has various activities (antiarrhythmic, antiasthmatic, antiherpetic, and antitussive activity) (Duke, 1992). This compound is recognized as a bactericide, fungicide, and viricide in plant tissues (Walker and Stahmann, 1955). The difference in the peroxyl radical-scavenging activity of these compounds should be attributed to the functional groups at the C-1 position, namely, the carboxyl group and the aldehyde group. The electron attractive ability of the aldehyde group is stronger than that of carboxyl group, therefore, the former may reduce the hydrogen-donating ability of the phenolic hydroxyl groups more strongly than the latter.

Of the isolated flavonoids, vitexin and isovitexin revealed almost no peroxyl radical-scavenging activity. Bors et al. (1990) proposed that the presence of the 3′,4′-dihydroxy structure (catechol structure), 2,3-double bond, and 3- and 5-hydroxyl groups are important in the expression of the peroxyl radical-scavenging activity in the flavonoids. Quercetin has all the above mentioned structures; rutin and hyperin do not have a 5-hydroxyl group, whereas vitexin and isovitexin do not have the above structures. However, the isolation of isovitexin from long-life rice seeds (Ramarathnam et al., 1989) suggests that this compound probably acts as a protectant in the seeds against oxidative damage.

Moreover, we obtained two fractions (G and H) containing proanthocyanidins by Sephadex LH-20 chromatography. The main constituents of fraction G were proanthocyanidins mainly with catechin and/or epicatechin and afzelechin and/or epiafzelechin as extender units, whereas fraction H contained a greater amount of constituents other than procyanidins with catechin and/or epicatechin as extender units. Proanthocyanidins (condensed tannins) are widely distributed in fruits and vegetables and are thought to act as a chemical defense system against insects, animals, birds, and reptiles by their protein-adsorbing capacity and astringency (Harborne, 1988). In addition, procyanidin has various activities, such as the antihuman immunodeficiency virus (anti-HIV) activity (Duke, 1992). Because proanthocyanidins are different in their biological activities due to their molecular weight (Ohara, 1994; Ohara et al., 1994), it is expected that fraction G, which contains various degrees of polymerization of the proanthocyanidins, also exhibits various activities.

The result of the peroxyl radical-scavenging activity of the isolated compounds at the same concentration indicates that the reactivity of the products generated by termination with these compounds and peroxyl radicals is not identical. The fact that all of the isolated active compounds have dihydroxy groups shows that this structure is useful for peroxyl radical-scavenging activity not only in flavonoids but also in other compounds. The radicals, which are generated by releasing the hydrogen radical from the dihydroxy groups of the

isolated compounds, will generate secondary oxidized products by reaction with peroxyl radicals and other oxidized products. The oxidized products derived from protocatechuic acid, the decrease of whose peroxyl radical scavenging activity during the experiment was the least in the isolated compounds at the same concentration, may be stable and still have peroxyl radical-scavenging activity. In contrast, the induction period of rutin was shorter than that of protocatechuic acid, and thereafter the induction period MeL-OOHs were increased more rapidly than control. Further study is required to analyze the stability and peroxyl radical-scavenging activity of those oxidized products.

The contents of the isolated antioxidant compounds and fractions (G and H) in the ethanolic extract from the hulls show that the peroxyl radical-scavenging activity of the extract should be mainly attributed to proanthocyanidin fractions in the isolated antioxidant constituents. Besides this component, most of the activity of the extract would be explained by the contribution to the activity of quercetin, rutin, hyperin, protocatechuic acid, and 3,4-dihydroxybenzaldehyde. It may well be assumed that the groat of buckwheat is protected against oxidative damage by its hulls with these antioxidant constituents. However, the main constituent in the active fraction H is superficial, and we should identify and quantify it. Besides this, we will investigate the utilization of the antioxidant constituents in buckwheat hulls and their difference among genetic resources.

We are now trying to isolate antioxidant compounds also from buckwheat groats. The investigation of antioxidant compounds in buckwheat groats will be useful for the breeding of new varieties which are good for health by effectively preventing the synthesis of peroxyl radicals from polyunsaturated fatty acids.

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.

ACKNOWLEDGMENT

We thank Mr. K. Ito, Tohoku National Agricultural Experiment Station, for help in preparing the powdered samples of buckwheat hulls.

LITERATURE CITED

Bors, W.; Heller, W.; Michel, C.; Saran, M. Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. In *Methods in Enzymology*; Packer, L., Glazer, A. N., Eds.; Academic Pres: New York, 1990; Vol. 186, pp 343−355.

Duke, J. A. In *Handbook of biologically active phytochemicals and their activities*; CRC Press: Boca Raton, FL, 1992; pp 134.

Durkee, A. B. Polyphenols of the bran-aleurone fraction of buckwheat seed (*Fagopyrum sagitatum*, Gilib). *J. Agric. Food Chem.* **1977**, *25*, 286−287.

Harborne, J. B. In *Introduction to ecological biochemistry*; Academic press: London, 1988; pp 164.

Mabry, T. J.; Markham, K. R.; Thomas, M. B. The ultraviolet spectra of flavones and flavonols. In *The systematic identification of flavonoids*; Springer-Verlag: New York, 1970; Chapter 5.

Margna, U. V.; Margna, É. R. Differential nature of quantitative shifts in flavonoid accumulation in buckwheat seedlings of different ages. *Sov. Plant Physiol.* **1982**, *29*, 223−230.

Markham, K. R. Ultraviolet−visible absorption spectroscopy. In *Techniques of flavonoid identification*; Academic Press: London, 1982; pp 36−51.

Mulvena, D.; Webb, E. C.; Zerner, B. 3,4-Dihydroxybenzaldehyde, a fungistatic substance from green cavendish bananas. *Phytochemistry* **1969**, *8*, 393−395.

Nakatani, N. Recent advances in the study on natural antioxidants (in Japanese). *J. Jpn. Soc. Food Sci. Technol.* **1990**, *37*, 569−576.

Ohara, S. Chemistry and utilization of condensed tannins from tree barks. *JARQ* **1994**, *28*, 70−78.

Ohara, S.; Suzuki, K.; Ohira, T. Condensed tannins from *Acacia mearnsii* and their biological activities. *Mokuzai Gakkaishi* **1994**, *40*, 1363−1374.

Osawa, T.; Ramarathnam, N.; Kawakishi, S.; Namiki, M.; Tashiro, T. Antioxidative defense systems in rice hull against damage caused by oxygen radicals. *Agric. Biol. Chem.* **1985**, *49*, 3085−3087.

Osawa, T.; Ramarathnam, N.; Kawakishi, S.; Namiki, M. Antioxidative defense systems generated by phenolic plant constituents. In *Phenolic compounds in food and their effects on health II*; Huang, M.-T.; Ho, C.-T., Lee, C. Y., Eds.; American Chemical Society: Washington, DC, 1992; Chapter 10.

Porter, L. J.; Hrstich, L. N.; Chan, B. G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* **1986**, *25*, 223−230.

Ramarathnam, N.; Osawa, T.; Namiki, M.; Kawakishi, S. Chemical studies of novel rice hull antioxidants. 2. Identification of isovitexin, a C-glycosyl flavonoid. *J. Agric. Food Chem.* **1989**, *37*, 316−319.

Ribereau-Gayon, P. In *Plant phenolics*; Oliver and Boyd: Edinburgh, 1972; p 184.

Sato, H.; Sakamura, S. Isolation and identification of flavonoids in immature buckwheat seed (*Fagopyrum esculentum* Mönch) (in Japanese). *Agric. Chem. Soc. Jpn.* **1975**, *49*, 53−55.

Terao, J.; Karasawa, H.; Arai, H.; Nagao, A.; Suzuki, T.; Takama, K. Peroxyl radical scavenging activity of caffeic acid and its related phenolic compounds in solution. *Biosci. Biotech. Biochem.* **1993**, *57*, 1204−1205.

Terao, J.; Matsushita, S. The peroxidizing effect of α-tocopherol on autoxidation of methyl linoleate in bulk phase. *Lipids* **1986**, *21*, 255−260.

Walker, J. C.; Stahmann, M. A. Chemical nature of disease resistance in plants. *Annu. Rev. Plant Physiol.* **1955**, *6*, 351−366.

Watanabe, M.; Sato, A.; Osawa, R.; Terao, J. Antioxidative activity of buckwheat seed extracts and its rapid estimate for evaluation of breeding materials (in Japanese with English summary). *J. Jpn. Soc. Food Sci. Technol.* **1995**, *42*, 649−655.

Received for review July 26, 1996. Revised manuscript received February 5, 1997. Accepted February 6, 1997.[⊗]

JF9605557

[⊗] Abstract published in *Advance ACS Abstracts*, March 15, 1997.