A Novel Antioxidant Isolated from Young Green Barley Leaves

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An ethanol extract obtained from freeze-dried young green barley leaves was fractionated with column chromatography. The fraction exhibiting strongest antioxidative activity was further purified by HPLC. The active component was identified as 2″(3″)-*O*-glycosylisovitexin by gas chromatography, UV, FAB-MS, ^{1}H NMR, and ^{13}C NMR. Its antioxidative activity was almost equivalent to that of α-tocopherol in a lipid peroxidation system at the level of 100 μg/1.5 mg of ethyl linoleate.

Recently, natural plants have received much attention as sources of biologically active substances including antioxidants, antimutagens, and anticarcinogens.

Antioxidants are scavengers of oxygen radicals or hydroxy radicals that have been proposed to be agents that attack polyunsaturated fatty acids in cell membranes, giving rise to lipid peroxidation (Aust and Sringen, 1982). Lipid peroxidation is strongly associated with aging and carcinogenisis (Yagi, 1987; Harman, 1982; Cutler, 1984). Even though living systems are protected from active oxygen species by enzymatic inactivation systems, dietary antioxidants such as α-tocopherol and ascorbic acid may be effective in protection from peroxidative damage.

Synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene have been used as antioxidants for foods since the beginning of this century. The use of these synthetic antioxidants, however, has begun to be restricted because of their toxicity (Branen, 1975; Ito et al., 1983). Therefore, the importance of natural antioxidants has increased greatly.

There is a pressing need to find safe, economic antioxidants to replace these synthetic chemicals. In the present study, a chemical possessing antioxidative activity was isolated and identified from an extract of young green barley, which reportedly contains certain materials with anti-inflammatory activity (Matsuoka et al., 1983).

EXPERIMENTAL PROCEDURES

Chemicals. Surfactant, sodium dodecyl sulfate (SDS), hydrogen peroxide, thiobarbituric acid (TBA), malonaldehyde bis(diethyl acetal), and α-tocopherol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Ethyl linoleate, Trizma-HCl [tris(hydroxymethyl)aminomethane hydrochloride], and butylated hydroxytoluene (BHT) were obtained from Sigma Chemical Co. (St. Louis, MO).

Barley Leaves (*Hordium vulgare* L. Var. *nudum* Hook). Green barley leaves (110 g) were harvested 2 weeks after germination. The barley leaves were freeze-dried for 3 days in a freeze-dryer, Model 50-SRC-5 (Virtis Co., Gardiner, NY). The freeze-dried leaves were subsequently ground with a Wiley mill Standard Model 3 (Arthur H. Thomas Co., Philadelphia, PA) equipped with a mesh size 2-mm sieve to form a fine and uniform powder for extraction.

Sample Preparations. The freeze-dried powder (20 g) was placed in a glass column (4.5 cm × 40 cm), and a 500-mL portion of *n*-hexane was poured through the column twice. Subsequently, the column was eluted twice with a 500-mL portion of 80% ethanol. After the hexane and ethanol eluates were filtered, the solvents were removed in vacuo using a rotary flash evaporator. Approximately 1.1 and 13 g of pasty materials were obtained from the hexane and ethanol eluates, respectively.

The ethanol eluate (13 g) was separated into 11 fractions by a 40 cm × 4.5 cm i.d. glass column packed with Amberlite XAD-2 nonionic polymeric absorbent (Aldrich) according to the conditions shown in Table I, which also displays the amounts of materials obtained from each fraction after removal of solvent.

Antioxidative Activity Testing. Ethyl linoleate was oxidized by Fenton's reagent (Fe^{2+}/H_2O_2) according to the method described in Tamura et al. (1991). A hexane eluate (0.5 mg), an ethanol eluate (0.5 mg), and fractions (0.5 mg) from the ethanol eluate were added to 5 mL of an aqueous solution containing ethyl linoleate (1.5 mg/L), 0.25 mmol of Trizma-HCl/0.75 mmol of potassium chloride buffer (pH 7.4), 0.2% SDS, 1 μmol of ferrous chloride, and 0.5 μmol of hydrogen peroxide in a 20-mL test tube. α-Tocopherol (0.22 mg) was used as a comparative standard to evaluate the antioxidative activity of samples. The solutions were stirred for 16 h at 37 °C, and then 50 μL of 4% BHT ethanol solution was added to prevent further oxidation. The blank sample was prepared from the mixture containing all ingredients except Fenton's reagent and a testing sample. The control sample was prepared from the mixture containing all ingredients except a testing sample.

The degree of oxidation was measured by thiobarbituric acid (TBA) assay according to the method of Ohkawa et al. (1979). The testing solutions (0.2 mL) were mixed with 0.2 mL of aqueous 8% SDS, 1.5 mL of 1 M acetate buffer, and 1.5 mL of aqueous 0.67% TBA in a test tube with a stopper. The mixtures were heated at 90 °C for 60 min in a steam bath. After the solutions cooled to room temperature, 5 mL of saturated aqueous butanol solution was added. The solutions were centrifuged at 3000 rpm for 20 min, and then the absorbance of the upper butanol layer was measured at 532 nm with an HP 8452 diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA). The calibration curve for the TBA–MA adduct was prepared using standard MA obtained from hydrolysis of malonaldehyde bis(diethyl acetal).

Purification of the Active Compound (I). The most active fraction (fraction IV, crude compound I) was recrystallized with cold methanol twice. A light yellow powder was obtained. The purity of this material was 96% as measured by an HPLC equipped with a 25 cm × 4.6 mm i.d. Develosil ODS 5 column. Methanol was used as a solvent at flow rate of 1 mL/min. Further purification of this material was conducted with a preparative HPLC using a 25 cm × 1 cm i.d. Develosil ODS-5 column and a UV detector at λ = 280 nm. The solvent system was methanol/water (3/7) at flow rate of 2.5 mL/min. Approximately 6.5 mg of purified compound I (100%) was obtained from 10 mg of crude sample. Antioxidative activity of crude and purified compound I was determined according to the method described above.

Analysis of Compound I. The fast atom bombardment mass

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Table I. Amounts of Eluates Recovered from Column Chromatographic Fractions

fraction	solvent (1 L)	solvent ratio	amt of eluate, mg
I	water	100	4770
II	water/methanol	80/20	180
III	water/methanol	60/40	131
IV	water/methanol	40/60	199
V	water/methanol	20/80	32
VI	methanol	100	165
VII	acetone	100	870

spectra (FAB-MS) of compound I were recorded on a JEOL JMS-DX-705L with glycerol as the mounting matrix. ^1H NMR and ^{13}C NMR spectra were obtained by a JEOL JNM-EX-270 NMR instrument (270 MHz for ^1H and 67.5 MHz for ^{13}C) in DMSO-d_6 for all samples except for an aglycon (in CD_3OD). IR was recorded on a Jasco FT/IR-8300 with KBr. UV was obtained with a Jasco Ubest-50 in methanol ($C = 2.15 \times 50^{-5}$ M). Specific rotation was measured by a Jasco DIP-370 spectrometer in methanol (C = 0.64%).

To confirm the structure of compound I, it was hydrolyzed in an acidic solution. Compound I (9.5 mg) was dissolved in a 5% methanol solution of HCl (5 mL). The mixture was refluxed for 2 h in a water bath. After the reaction solution was cooled to room temperature, 5 mL of deionized water was added, and then the solution was extracted twice with 5-mL portions of ethyl acetate. After the solution was dried over anhydrous sodium sulfate, the solvent was removed in vacuo. The aglycon of compound I was isolated from the residue using preparative thin-layer chromatography (TLC, Merck silica plate SI-60 F_{254}, d_f = 0.5 mm). The solvent system was chloroform/methanol/water (60/30/10). A crude aglycon was further purified using an HPLC equipped with a 25 cm × 4.6 mm i.d. Develosil ODS-5 column and a UV detector (λ = 280 nm), from which 2.5 mg of a pure aglycon was obtained. The solvent system was methanol/water (40/60).

The remaining aqueous layer from ethyl acetate extraction was freeze-dried. The resulting dried sugar fraction was dissolved in 0.5 mL of dry pyridine and heated with 0.3 mL of hexamethyldisilazane (HMDS) and 0.1 mL of trimethylchlorosilane (TMCS) for 2 min at 100 °C. The methylated sugar fraction was analyzed with a gas chromatograph (Shimadzu GC-9A) equipped with a 2 m × 3 mm i.d. glass column packed with silicon GE SE-52 (3%) coated on Chromosorb W and a flame ionization detector. The isothermal oven temperature was 180 °C. Detector and injector temperatures were 230 °C.

Antioxidative Testing on Crude and Pure Compound I. Antioxidative activity of crude and pure compound I was determined using the method described above. The concentrations of samples tested were from 0 to 100 μg/mL of testing solution (100 μg/1.5 mg of ethyl linoleate). α-Tocopherol was used as positive control for antioxidative activity testing.

RESULTS AND DISCUSSION

The antioxidative activities of the samples are shown in Figure 1. n-Hexane and H_2O (fraction I) eluates exhibited prooxidative activity, suggesting the presence of prooxidants. On the other hand, all samples except those two exhibited certain degrees of antioxidative activity. Fraction IV, which is a methanol/water (60/40) eluate, showed the strongest antioxidative activity. It inhibited lipid peroxidation to over 90%, which is almost equivalent to the inhibition shown by the known natural antioxidant α-tocopherol under the test system used in the present study.

Characterization of Compound I. The UV absorption of compound I showed characteristic spectra of flavonoid: λ_{max} in methanol 272 nm (ϵ = 21 400) and 335 (ϵ = 24 900); specific rotation $[\alpha]_D$ was −41.7°.

The IR spectrum showed absorptions at 3420, 1620, 1490, 1194, and 1092 cm^{-1}. A broad peak at 3420 cm^{-1} is due to O–H stretching mode of the alcohol or the phenol. The

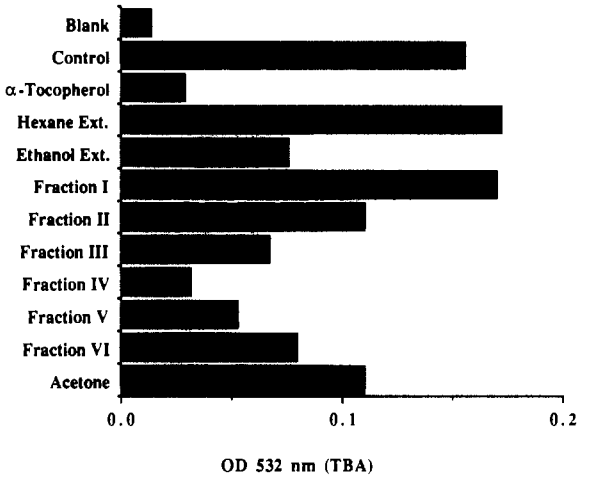

Figure 1. Antioxidative activities of samples obtained from green barley extract.

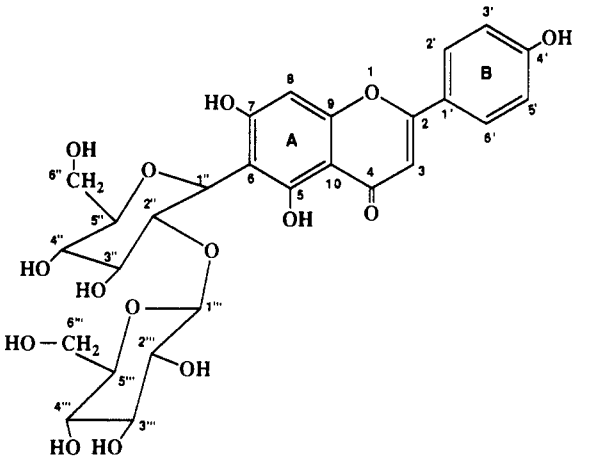

Figure 2. Structure of compound I. (See Table II for ^{13}C NMR chemical shift assignment of carbon atoms.)

characteristic absorption observed at 1620 cm^{-1} suggests the presence of a pyrone ring (Szymanski, 1963).

The FAB-MS of compound I gave an M + 1 ion peak at 595, suggesting that the molecular weight is 594.

On the basis of mass spectral data and the characteristic nature of the UV spectrum, compound I was proposed to have an isovitexin moiety, as was reported by Ramarathnam et al. (1989). In fact, most spectral data match those of authentic isovitexin (MW = 432). The structure of compound I is elucidated in Figure 2 (2″-O-glycosylisovitexin).

The ^1H NMR showed the aromatic protons on the B ring at δ 692 (H-3′ and H-5′, d, J = 9 Hz) and 7.97 (H-2′ and H-6′, d, J = 9 Hz) and those on the A ring at δ 6.88 (H-3, s) and 6.96 (H-8, s). The glucosyl H-1″ and H-1‴ are at δ 4.65 and 5.00 (d, J = 6.9 Hz), respectively. The remaining glucosyl protons appeared in the range δ 3.20–4.00. These data suggest that H-1″ at δ 4.65 is the C–C glycosidic linkage and that H-1‴ at δ 5.00 is O-glycosidic β-linkage.

Table II shows ^{13}C NMR of compound I, along with assignment of each chemical shift. Compound I gave signals of 27 carbon atoms. An empirical formula of compound I was elucidated as $C_{27}H_{30}O_{15}$ on the basis of the FAB-MS and ^{13}C NMR. It represents a flavonoid diglycoside with C–C and C–O glycosidic linkages. Chemical shifts of 20 of 27 carbons are consistent with those of authentic isovitexin (Ramarathnam et al., 1989). The two-dimensional NMR spectra, ^1H–^1H COSY and ^{13}C–^1H COSY, support the presence of O-glycosylisovitexin.

Table II. Assignment of Chemical Shifts in the ^{13}C NMR Spectra of Compound I

δ	assignment	δ	assignment	δ	assignment
182.1 (s)	C-4	116.1 (d)	C-3′ and C-5′	77.2 (d)	C-5‴
164.2 (s)	C-2	110.6 (s)	C-6	75.8 (d)	C-3‴
162.5 (s)	C-7	104.9 (s)	C-10	73.8 (d)	C-2‴
161.5 (s)	C-4′	103.2 (d)	C-3	72.7 (d)	C-1″
159.4 (s)	C-5	101.2 (d)	C-1‴	70.9 (d)	C-2″
156.4 (s)	C-9	93.8 (d)	C-8	69.6 (d)	C-4″ or C-4‴
128.7 (d)	C-2′ and C-6′	81.0 (d)	C-5″	69.5 (d)	
120.9 (d)	C-1′	78.9 (d)	C-3″	60.7 (d) 60.3 (d)	C-6″ or C-6‴

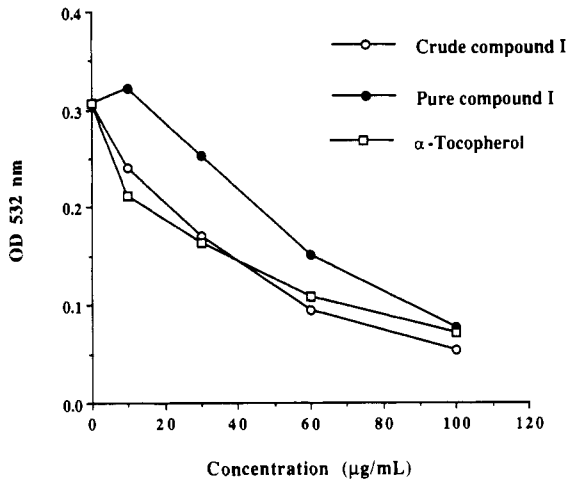

Figure 3. Antioxidative activities of crude and pure compound I.

After compound I was hydrolyzed into an aglycon and a sugar, the aglycon part was identified as isovitexin by comparison of ^1H NMR and ^{13}C NMR spectra of authentic isovitexin. The GC retention time of the trimethylsilane (TMS) derivative of the sugar part was 9.09 min, which exactly matches that of an authentic TMS derivative of methyl glucoside. These results suggest that compound I is *O*-glycosylisovitexin with a β-linkage connected between isovitexin and glucose at 2″-O or 3″-O. It is not clear whether compound I is 2″-*O*- or 3‴-*O*-glucosylisovitexin. However, the 2″-O form is more likely because all glycosyl flavons reported in natural plants to date are in 2″-O form (Tschesche and Struckmeyer, 1976; Chaboud et al., 1986).

Antioxidative Activity of Compound I (2″-*O*-Glycosylisovitexin). Figure 3 shows the results obtained from antioxidative testing of compound I. The crude compound I showed activities almost equivalent to those of α-tocopherol. The pure compound I exhibited slightly less activity than did the crude one within the range of concentration tested. However, three samples showed almost equal antioxidative activities at the level of 100 μg/1.5 mg of ethyl linoleate.

Conclusion. Flavonoids occur through the entire plant kingdom, from fungi to angiosperms. They occur as aglycons and glycosides. The preferred bonding site of the sugar residue to the flavonol is the 3-position (much less frequently the 7-position). *C*-Glycosyl flavonoids, in which the sugar moiety is attached by a carbon–carbon bond directly to the aromatic ring, are also reported to occur predominantly in the leaves of various cereal crops as derivatives of apigenin and luteolin (Herrmann, 1976). Vitexin, a glucose derivative of apigenin, is the best known of this group (Robinson, 1983). Ramarathnam et al. (1989) isolated and identified isovitexin in rice hull. They also reported that the antioxidative activity of the isovitexin-containing fraction isolated from rice hull was as strong as that of α-tocopherol.

Many flavonoids and their related compounds have been known to possess strong antioxidative characteristics (Pratt, 1979; Pratt and Birac, 1979). Their antioxidative activity is due to their ability to chelate metal ions by means of either the 3-hydroxy/4-keto grouping or the 5-hydroxy/4-keto grouping and their ability to scavenge free radicals derived from the phenolic moiety of the structure (Pratt, 1976; Dziedzic and Hudson, 1983). In addition to antioxidative activity, flavonoids have been known to possess high pharmacological potencies, including estrogenic effects for mammals (Lyman et al., 1959), anti-inflammatory effects (Baumann et al., 1982; Alcaraz and Villar, 1989), antiallergic effects (Gabor, 1986), antiviral activity (Selway, 1986), and possible anticarcinogenic effects (Glusker and Rossi, 1986).

It is possible to obtain 2″-*O*-glycosylisovitexin from green barley leaves in fairly large amounts by a simple method. Therefore, once this compound is proved not to be hazardous, it may be usable as an antioxidative agent in certain foods or beverages.

LITERATURE CITED

Alcaraz, M. J.; Villar, A. Anti-inflammatory effects of natural phenolic compounds. In *CRC Handbook of Free Radicals and Antioxidants in Biomedicine*; Miquel, J., Ed.; CRC Press: Boca Raton, FL, 1989; Vol. II, pp 77–85.

Aust, S. D.; Sringen, B. A. The role of ion in enzymatic lipid peroxidation. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: Orlando, 1982; Vol. 5, Chapter 1.

Baumann, J.; Bruchhausen, F. V.; Wurm, G. Flavonoids and arachidonic acid metabolism. In *Flavonoids and Bioflavonoids*; Farkas, L., Gabor, M., Kallay, F., Wagner, H., Eds.; Elsevier Scientific: Amsterdam, 1982; pp 411–419.

Branen, A. L. Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. *J. Am. Oil Chem. Soci.* **1975**, *52*, 59–63.

Chaboud, A.; Raynaud, J.; Debourcieu, L. Presence of 2″-O-glucosyl 6-C-glucosyl apigenin or 2″-O-glucosylisovitexin in leaves of Thea chinensis *Sims var. macrophylla Sieb.* (Yunnan Tuocha tea). *Pharmazie* **1986**, *41*, 745–746.

Cutler, R. G. Antioxidants, aging, and longevity. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: Orlando, 1984; Vol. 6, Chapter 11.

Dziedzic, S. Z.; Hudson, B. J. F. Hydroxy isoflavones as antioxidants for edible oils. *Food Chem.* **1983**, *11*, 161–166.

Gabor, M. Anti-inflammatory and anti-allergic properties of flavonoids. In *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships*; Cody, V., Middleton, E., Jr., Harborne, J. B., Eds.; Liss: New York, 1986; pp 471–480.

Glusker, J. P.; Rossi, M. Molecular aspects of chemical carcinogens and bioflavonoids. In *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships*; Cody, V., Middleton, E., Jr., Harborne, J. B., Eds.; Liss: New York, 1986; pp 395–410.

Harman, D. The free-radical theory of aging. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: Orlando, 1982; Vol. 5, Chapter 8.

Herrmann, K. Flavonols and flavones in food plants: A review. *J. Food Technol.* **1976**, *11*, 433–448.

Ito, N.; Fukushima, S.; Hasegawa, A.; Shibata, M.; Ogiso, T. Carcinogenicity of butylated hydroxyanisole in F344 rats. *J. Natl. Cancer Inst.* **1983**, *70*, 343–347.

Lyman, R. L.; Bickoff, E. M.; Booth, A. N.; Livingston, A. L. Detection of coumestrol in leguminous plants. *Arch. Biochem. Biophys.* **1959**, *80*, 61–67.

Matsuoka, Y.; Seki, H.; Kubota, K.; Otake, H.; Hagiwara, Y. Anti-inflammatory effect of glycoproteins, D 1-G 1, separated from barley leaves. *Ensho* **1983**, *3*, 602–604.

Ohkawa, H.; Ohishi, N.; Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **1979**, *95*, 351–358.

Pratt, D. E. Role of flavones and related compounds in retarding lipid oxidative flavor changes in foods. In *Phenolic, Sulfur, and Nitrogen Compounds in Food Flavors*; Charalambous, G., Kats, I., Eds.; American Chemical Society: Washington, DC, 1976; pp 1–13.

Pratt, D. E. Natural antioxidants of soybeans and other oil-seeds. In *Autooxidation in Food and Biological Systems*; Simic, M. G., Karel, M., Eds.; Plenum Press: New York, 1979; pp 283–293.

Pratt, D. E.; Birac, P. M. Source of antioxidant activity of soybeans and soy products. *J. Food Sci.* **1979**, *44*, 1720–1722.

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

Robinson, T. Flavonoids and related compounds. In *The Organic Constituents of Higher Plants: Their Chemistry and Interrelationships*; Burgess Publishing: Minneapolis, 1963; pp 173–240.

Selway, J. W. T. Antiviral activity of flavones and flavans. In *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships*; Cody, V., Middleton, E., Jr., Harborne, J. B., Eds.; Liss: New York, 1986; pp 521–536.

Szymanski, A. *Infrared Band Handbook*; Plenum Press: New York, 1963; p 118.

Tamura, T.; Kitta, K.; Shibamoto, T. Formation of reactive aldehydes from fatty acids in a Fe^{2+}/H_2O_2 oxidation system. *J. Agric. Food Chem.* **1991**, *39*, 439–442.

Tschesche, R.; Struckmeyer, K. On 2″-gluco-isovitexin from wood sorrel (*Oxalis acetosella L.*). *Chem. Ber.* **1976**, *109*, 2901–2907.

Yagi, K. Lipid peroxides and human disease. *Chem. Phys. Lipids* **1987**, *45*, 337–341.

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