Antioxidative Substances in Leaves of *Polygonum hydropiper*

Hiroyuki Haraguchi,*,† Kensuke Hashimoto,† and Akira Yagi‡

Faculty of Engineering and Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Higashimura-cho, Fukuyama 729-02, Japan

Antioxidative flavonoids and flavonoid glucoside were isolated from the leaves of *Polygonum hydropiper*, a culinary herb. The flavonoid Ia was identified as 7,4′-dimethylquercetin, Ib as 3′-methylquercetin, Ic as quercetin, and the flavonoid glucoside Id as isoquercitrin on the basis of chemical and spectroscopic evidence. The antioxidative activities measured by a ferric thiocyanate method were in the order Id > Ia > Ic > Ib.

INTRODUCTION

Recently, several attempts to replace synthetic antioxidants for the natural ones have been developed. As resources of natural antioxidants for protection against the rancidity of fats and oils in food, much attention has been paid to plants (Larson, 1988; Zhang et al., 1990), algae (Fujimoto and Kaneda, 1980; Tutour, 1990), and microbial products (Aoyama et al., 1982; Zaika and Smith, 1975). Especially, the antioxidative compounds present in edible plants have recently been received as food additives (Fukuda et al., 1985; Igarashi et al., 1990) because of a toxic side effect in synthetic antioxidants (Hashek and Witschi, 1979).

Polygonum hydropiper L. is used as a medicinal herb against cancer (Hartwell, 1970) and hemostatics (Steinberg, 1928). The young shoot is edible as a spice with raw fish in Japan. Lipophilic materials of this plant have various biological activities, polygodial and warburganal possessing antifungal activity (Taniguchi et al., 1983, 1988) and polygonolide possessing anti-inflammatory activity (Furuta et al., 1986). Hydrophilic flavonoids, such as rhamnazin and persicarin, were isolated, but no study on biological activity has been reported so far (Gottlieb, 1975). During the course of our study on bioactive metabolites of this plant, we found three flavonoids and a flavonoid glucoside from the water-soluble fraction of the leaf extract as antioxidant principles. In this paper, we report the structures and the antioxidative activity of these flavonoids.

EXPERIMENTAL PROCEDURES

Materials and Equipment. Leaves of *P. hydropiper* grown near the Ashida River (Fukuyama, Japan) were collected in June 1990. UV spectra were obtained with a Shimadzu UV-260 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on JEOL SP-100 (100 MHz) and FX-100 (25 MHz) spectrometers, respectively. Chemical shifts are in parts per million from TMS as an internal standard. Mass spectra were recorded with a Shimadzu GC-MS 6020 spectrometer at 70 eV. Analytical TLC was carried out on Merck Kieselgel 60 F_{254}, and visualization was done by placing the plate in a chamber containing I_2 vapor and by heating with aniline hydrogen phthalate reagent for sugars. TLC solvents are the following: $CHCl_3$–MeOH (9:1) and $CHCl_3$–MeOH–AcOH (7:3:1) for flavonoids and BuOH–pyridine–H_2O (6:4:3) for sugars.

Extraction and Isolation. Fresh leaves (1.8 kg) of *P. hydropiper* were extracted three times with MeOH (3 L each) at room temperature. The combined MeOH extract was concentrated on a rotary evaporator to yield a green mass (120 g). The dried extract was partitioned between *n*-hexane and H_2O. The water-soluble layer was subjected to highly porous polymer (Diaion HP-20; particle size 75–150 μm; Mitsubishi Kasei Co. Ltd.) column chromatography (8 × 40 cm), which was successively eluted with water (5 L), 30% MeOH (3 L), and MeOH (3 L). The MeOH eluate was concentrated to dryness in vacuo. The residue (1.7 g) was dissolved in $CHCl_3$ and chromatographed on a silica gel (BW-820 MH; 70–200 mesh, Fuji Davison Chemical Co. Ltd.) column (3.2 × 50 cm), which was developed with $CHCl_3$ and 2, 5, and 10% MeOH in $CHCl_3$, successively. The fraction eluted with $CHCl_3$ gave Ia (74 mg) as yellow needles after concentration followed by recrystallization from MeOH. The fraction obtained from 10% MeOH eluate was rechromatographed on a silica gel column (2.8 × 40 cm) using a gradient elution of $CHCl_3$–MeOH. Recrystallization of the first eluate (fractions 5–7) from MeOH afforded Ib (25 mg) as pale yellow powders and the second eluate (fractions 16–18) Ic (12 mg) as yellow powders. The $CHCl_3$-insoluble materials obtained from the MeOH eluate of a Diaion HP-20 column gave Id (60 mg) after recrystallization from MeOH as pale yellow needles.

Compound Ia: yellow needles; mp 236–240 °C; UV λ_{max} (MeOH) 207.0 (log ε = 4.76), 254.0 (4.59), 265.0 (4.59), 265.0 (sh), 371.0 (4.60) nm; UV λ_{max} (MeOH–$AlCl_3$) 205.4, 264.4, 430.2 nm; UV λ_{max} (MeOH–NaOAc) 207.4, 254.6, 372.8 nm; UV λ_{max} (MeOH–$AlCl_3$/HCl) 206.2, 263.6, 361.8, 429.9 nm; UV λ_{max} (MeOH–H_3BO_3–NaOAc) 210.6, 254.6, 371.2 nm; ^{1}H NMR (DMSO-d_6) δ 3.86 (6 H, s, OCH_3 × 2), 6.34 (1 H, d, J = 2 Hz, C_6H), 6.78 (1 H, d, J = 2 Hz, C_8H), 6.90 (1 H, d, J = 8 Hz, $C_{5'}H$), 7.78 (1 H, dd, J = 8, 2 Hz, $C_{6'}H$), 7.79 (1 H, d, J = 2 Hz, $C_{2'}H$), 9.53 (1 H, s, OH), 9.76 (1 H, s, OH), 12.46 (1 H, s, C_5OH); ^{13}C NMR (DMSO-d_6) δ 175.9 (s, C4), 164.9 (s, C7), 160.4 (s, C5), 156.9 (s, C9), 148.9 (s, C2), 147.4 (s, C3′), 147.0 (s, C4′), 136.1 (s, C3), 121.9 (d, C6′), 121.9 (s, C1′), 115.5 (d, C5′), 111.8 (d, C2′), 104.0 (s, C10), 97.4 (d, C6), 91.9 (d, C8), 56.0 (q, CH_3O), 55.9 (q, CH_3O); MS m/z 330 (M^+, 100%), 287 (M – CO, –CH_3, 43%), 151, 149, 135.

Compound Ib: yellow powder, mp 305 °C; UV λ_{max} (MeOH) 204.4 (log ε = 4.61), 254.4 (4.26), 370.8 (3.68) nm; UV λ_{max} (MeOH–$AlCl_3$) 206.4, 264.2, 361.0, 429.6 nm; UV λ_{max} (MeOH–NaOAc) 202.0, 256.2, 273.2, 321.2, 380.4 nm; UV λ_{max} (MeOH–$AlCl_3$/HCl) 206.4, 263.0, 357.8, 428.4 nm; UV λ_{max} (MeOH–H_3BO_3–NaOAc) 213.2, 254.8, 373.4 nm; ^{1}H NMR (DMSO-d_6) δ 3.18 (3 H, s, OH), 3.85 (3 H, s, CH_3O), 6.18 (1 H, d, J = 2 Hz, C_6H), 6.46 (1 H, d, J = 2 Hz, C_8H), 6.95 (1 H, d, J = 8 Hz, $C_{5'}H$), 7.70 (1 H, dd, J = 8, 2 Hz, $C_{6'}H$), 7.75 (1 H, d, J = 2 Hz, $C_{2'}H$), 12.62 (1 H, s, C_5OH); ^{13}C NMR (DMSO-d_6) δ 175.4 (s, C4), 163.7 (s, C7), 160.2 (s, C5), 155.8 (s, C2 or C9), 148.4 (s, C2 or C9), 147.0 (s, C3′), 146.2 (s, C4′), 135.4 (s, C3), 121.7 (d, C6′), 121.4 (s, C1′), 115.2 (d, C5′), 111.3 (d, C2′), 102.6 (s, C10), 97.9 (d, C6), 93.4 (d, C8), 55.6 (q, CH_3O); MS m/z 316 (M^+, 12.9%), 314 (M – 2, 40.8%), 287 (M – CO, –H, 16.2%), 135.

Compound Ic: yellow powder; mp 310 °C; UV λ_{max} (MeOH) 204.1 (log ε = 4.56), 255.3 (4.11), 291.8 (sh), 370.0 (3.98) nm; mixed melting point of Ic with an authentic quercetin, 304 °C.

Compound Id: pale yellow needles; mp 242–243 °C; UV λ_{max}

* Author to whom correspondence should be addressed.

† Faculty of Engineering.

‡ Faculty of Pharmacy and Pharmaceutical Sciences.

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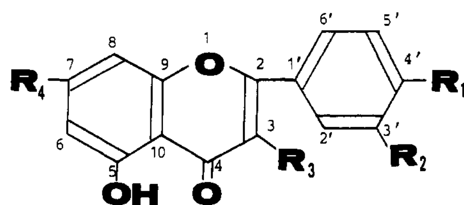

	R_1	R_2	R_3	R_4
Ia	OCH_3	OH	OH	OCH_3
Ib	OH	OCH_3	OH	OH
Ic	OH	OH	OH	OH
Id	OH	OH	O-Glc	OH

Figure 1. Structure of antioxidative components isolated from *P. hydropiper.*

(MeOH) 206.8 (log ε = 4.56), 256.0 (4.26), 359.6 (4.18) nm; UV λ_{max} (MeOH–$AlCl_3$) 209.4, 274.4, 427.4 nm; UV λ_{max} (MeOH–NaOAc) 207.2, 257.8, 268.0, 368.4 nm; UV λ_{max} (MeOH–$AlCl_3$/HCl) 209.0, 268.8, 298.4, 366.2, 402.4 nm; UV λ_{max} (MeOH–H_3BO_3–NaOAc) 213.8, 261.6, 300.4, 379.0 nm; ^1H NMR (DMSO-d_6) δ 3.0–3.6 (sugar CH), 5.37 (1 H, d, J = 7 Hz, anomeric H), 6.17 (1 H, d, J = 2 Hz, C_6H), 6.35 (1 H, d, J = 2 Hz, C_8H), 6.80 (1 H, d, J = 8 Hz, $C_{5'}H$), 7.60 (1 H, d, J = 2 Hz, $C_{2'}H$), 7.80 (1 H, dd, J = 8, 2 Hz, $C_{6'}H$), 12.5 (1 H, s, C_5OH); mixed melting point of Id with an authentic isoquercitrin, 241–242 °C. Hydrolysis of Id: Id (14 mg) in H_2O (1 mL) was hydrolyzed with β-glucosidase (1 mg) at room temperature for 24 h. From the hydrolysate, quercetin and glucose were identified by TLC.

Antioxidative Assay. Different amounts of samples dissolved in 120 μL of EtOH were added to a reaction mixture in a screw-cap vial. Each reaction mixture consisted of 2.88 mL of 2.51% linoleic acid in EtOH and 9 mL of 40 mM phosphate buffer (pH 7.0). The vial was placed in a oven at 40 °C in the dark. At intervals during incubation, a 0.1-mL aliquot of the mixture was diluted with 9.7 mL of 75% EtOH, which was followed by adding 0.1 mL of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance at 500 nm was measured (Nakatani et al., 1987).

RESULTS AND DISCUSSION

The methanol extract of *P. hydropiper* was partitioned between *n*-hexane and water. The water-soluble part showed strong antioxidative activity against linoleic acid in the ferric thiocyanate method. This fraction was further purified to obtain four active compounds: 7,4′-dimethylquercetin (Ia), 3′-methylquercetin (Ib), quercetin (Ic), and isoquercitrin (Id) (Figure 1). Figure 2 summarizes the purification processes of these flavonoids.

The ^1H NMR spectrum of compound Ia, mp 236–240 °C, showed the typical chemical shift due to the protons of flavonoid having two methoxy groups. This finding was supported by the parent peak of mass spectrum at m/z 330. The position of two methoxy groups in compound Ia was determined at C_7 and $C_{4'}$ by UV and ^{13}C NMR spectral examination (Wagner et al., 1976) and comparison with literature data (Anand et al., 1956).

The ^1H NMR spectrum of compound Ib, mp 305 °C, showed chemical shift of the protons similar to those of compound Ia. The position of a methoxy group in compound Ib was determined at $C_{3'}$ by UV and ^{13}C NMR spectral examination and comparison with literature data (Heap and Robinson, 1926).

The structure of compound Ic, mp 310 °C, was determined by UV spectral examination and direct mixed melting point to be quercetin.

The ^1H NMR spectrum of compound Id, mp 242–243 °C, showed the typical chemical shift due to flavonoid

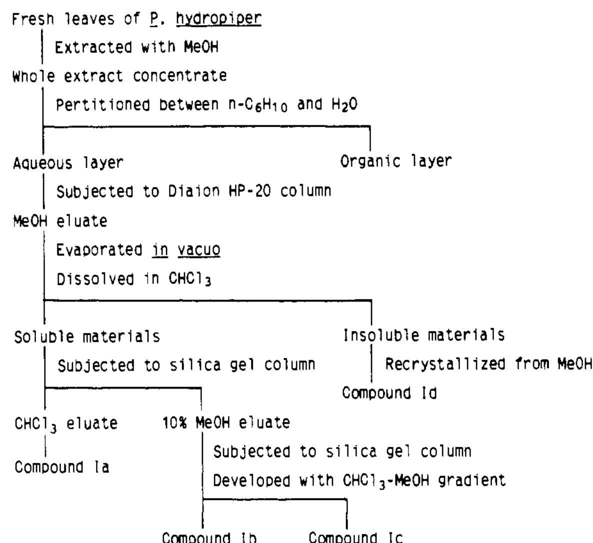

Figure 2. Isolation of antioxidative components from *P. hydropiper.*

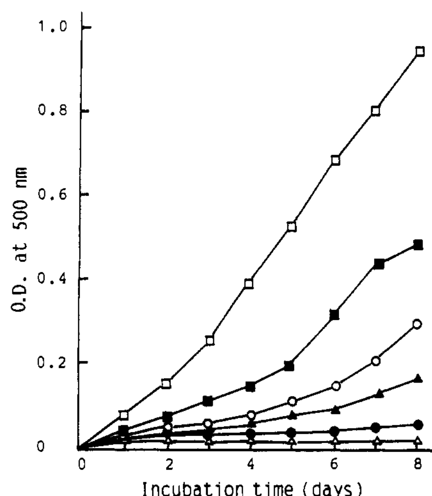

Figure 3. Antioxidative activity of flavonoids isolated from *P. hydropiper*. Each compound was added at a final concentration of 10 ppm based on the total volume of solution: (●) Ia; (○) Ib; (▲) Ic; (△) Id; (■) α-tocopherol; (□) control.

glycoside having an anomeric proton coupling constant, J = 7 Hz (Strack et al., 1988). On enzymatic hydrolysis compound Id showed glucose and quercetin by TLC analysis. The sugar position in compound Id was determined by UV spectral examination to be C_3. Thus, the structure of compound Id was established to be isoquercitrin to which no depression of mixed melting point was observed.

The antioxidative activity of these flavonoids was evaluated by a ferric thiocyanate method. Figure 3 shows that all of them were more active than α-tocopherol, a common natural antioxidant. Among them, both compounds Ia and Id revealed especially potent antioxidative activity. Concentration effects of Ia and Id on peroxidation of linoleic acid are shown in Figure 4, and compound Ia inhibited the oxidation of linoleic acid about 40% at 3 ppm. Compound Id was the most effective; almost complete inhibition was observed at 3 ppm. The ID_{50} values of Ia, Ib, Ic, and Id for oxidation of linoleic acid at day 8 were 1.5, 3.0, 2.5, and 0.6 ppm, respectively.

Antioxidative activities of various flavonoids are well-known. As to flavonols, the relationship between the position of the hydroxy groups and the antioxidant activity has been discussed (Pratt, 1976). Quercetin is an effective

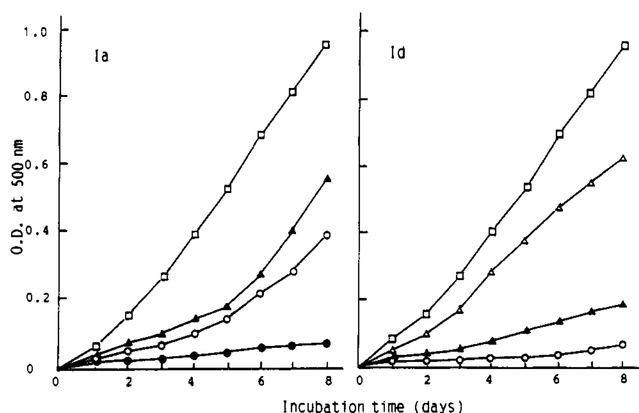

Figure 4. Concentration effects of antioxidative activity of compounds Ia and Id. The symbols show the final concentration of compounds Ia and Id added to the total reaction volume: (●) 10; (○) 3; (▲) 1; (△) 0.3; (□) 0 ppm.

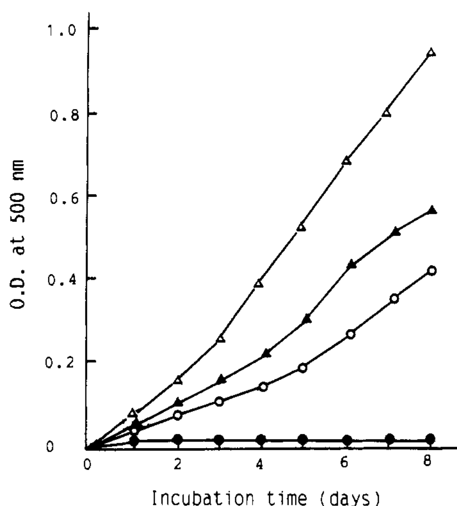

Figure 5. Antioxidative activity of quercetin glycosides. Each compound was added at a final concentration of 10 ppm based on the total volume of solution: (●) isoquercitrin; (○) quercitrin; (▲) rutin; (△) control.

flavonols in the same way as morin, kaempherol, and luteolin (Torel et al., 1986). Dimethylquercetins isolated from *P. hydropiper* were also effective on inhibition of lipid peroxidation. Especially 7,4′-dimethylquercetin (Ia) possessed a potent activity. Some 7-methylflavones isolated from *Thymus vulgaris* also showed strong antioxidative activity (Miura and Nakatani, 1989). This suggests that the position of the methoxy group in flavonoids participates in the antioxidant activity.

Isoquercitrin (Id) showed the most effective antioxidant activity among the flavonoids in *P. hydropiper*. Some quercetin glycosides are said to be effective inhibitors of the peroxidation reaction (Takahama, 1984). Figure 5 shows the evaluation of antioxidative activity of quercetin glycosides at 10 ppm. Isoquercitrin was obviously superior to rutin or quercitrin. Glycosidal pattern may be also deeply related with the antioxidant activity.

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Registry No. Ia, 529-40-8; Ib, 480-19-3; Ic, 117-39-5; Id, 522-12-3.