Metabolism of Rhaponticin and Chrysophanol 8-*o*-β-D-Glucopyranoside from the Rhizome of *Rheum undulatum* by Human Intestinal Bacteria and Their Anti-allergic Actions

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Rhaponticin and chrysophanol 8-*o*-β-D-glucopyranoside isolated from the rhizomes of *Rheum undulatum* (Family Polygonaceae) are metabolized to rhapontigenin and chrysophanol, respectively, by human intestinal microflora. Most intestinal bacteria isolated from human feces catalyzed these metabolic pathways. Among rhaponticin and chrysophanol 8-*o*-β-D-glucopyranoside and their metabolites, rhapontigenin had the most potent inhibitory activity on a hyaluronidase, a histamine release from mast cell and passive cutaneous anaphylaxis (PCA) PCA reaction. The inhibitory activity of rhapontigenin was more potent than that of disodium cromoglycate, one of the commercial anti-allergic drugs. These results suggest that rhaponticin in the rhizomes of *R. undulatum* is a prodrug that has an extensive anti-allergic property.

Key words rhapontigenin; antiallergic action; *Rheum undulatum*; intestinal bacteria; hyaluronidase

Most herbal medicines are orally administered, and most components of these medicines are inevitably brought into contact with intestinal microflora in the alimentary tract. Some were transformed by the intestinal bacteria before their absorption from the gastrointestinal tract.[1,2]

Hyaluronidase is one of the mucopolysaccharide-splitting enzymes, and is involved in the permeability of the vascular system and inflammation.[3,4] Kakegawa *et al.* reported that the anti-allergic drugs disodium cromoglycate (DSCG) and tranilast had a strong inhibitory effect on the activation of hyaluronidase, and also showed that hydrangenol derivatives inhibited not only the activation of hyaluronidase but also the release of histamine from rat peritoneal exudated cells induced by antigen IgE antibody reaction and compound 48/80.[5—7] Sakamoto *et al.* reported that DSCG, which suppressed the increase in capillary permeability caused by 48 h homologous passive cutaneous anaphylaxis (PCA) in rats, not only inhibited hyaluronidase *in vitro*, but also its activation induced by PCA.[8] These results seemed to indicate that potent hyaluronidase inhibitory substances might have anti-allergic effects, and could become leading compounds in the development of new anti-allergic drugs.

During the screening program to discover such compounds from natural products, *Rheun undulatum* (Family Polygonaceae) was found to show inhibitory activity on the activation of hyaluronidase. In this paper, we isolated some compounds from the rhizomes of *R. undulatum* (Rhei Rhizoma), and investigated their metabolism by human intestinal bacteria and the anti-allergic activities of their metabolites.

MATERIALS AND METHODS

Materials Hyaluronidase from bovine testis, compound 48/80, calcium ionophore A-23187, egg albumin, *p*-nitrophenyl β-D-glucopyranoside, Freund's complete adjuvant, anti-dinitrophenol (DNP)- IgE, DNP-human serum albumin (HSA), Evans blue and disodium cromoglycate (DSCG) were purchased from Sigma Chemical Co. (U.S.A.). Hyaluronic acid potassium salts, *o*-phthalaldehyde, trichloroacetic acid, histamine·2HCl and *Bordetella pertussis* vaccine were from Wako Pure Chemical Co. (Japan).

Instruments Melting points were determined on a Yanagimoto micromelting point apparatus. IR spectra were recorded on a Hitachi 260-01 spectrometer in KBr disks. UV spectra were obtained on a Shimadzu UV-2200 spectrometer. Electron impact-mass spectra (EI-MS; ionization voltage, 70 eV) were measured with a Finnigal Mat TSQ-700. ^{1}H- and ^{13}C-NMR spectra were taken on a Bruker AM-500 spectrometer with tetramethylsilane (TMS) as an internal standard. The TLC chromatograms were quantitatively analyzed with a Shimadzu CS-920 TLC scanner.

Extraction and Isolation of Anti-histamine Compounds from the Rhizome of *R. undulatum* Korean rhubarb rhizome (Rhizome of *R. undulatum*, Polygonaceae) cultivated in Deakwanryung, Kangwon-Do (Korea) was collected. The voucher specimen was deposited at the College of Pharmacy, Kyunghee University. The dried material (1 kg) was extracted three times in boiling water. The water extract was filtered and evaporated on a rotary evaporator under reduced pressure to obtain a viscous mass (160 g) of water extract. This material (30 g) was subjected to column chromatography on Silica-gel (Merck, Art. No. 7734). The column was eluted with $CHCl_3$–MeOH (10 : 1→10 : 4), and three fractions were collected. Crystallization of fraction 1 (20 mg) with MeOH yielded pure compound **1** (10 mg). Compounds **2** (250 mg) and **3** (65 mg) of fraction 2 (0.9 g) and fraction 3 (0.4 g) were isolated by silica gel column chromatography (elution solvents : $CHCl_3$–MeOH (20 : 1→20 : 5)), respectively.These compounds were identified by instrumental analysis.[9,10]

Chrysophanol (1) Yellow needles. mp 196—197 °C, IR ν_{max}^{KBr} cm^{-1} : 1676 (free C=O), 1626 (chelated C=O), 1560, 1540, 1456 (aromatic C=C). EI-MS (*m/z*) : 254 [M$^+$]. ^{1}H-NMR (DMSO-d_6) δ : 11.78 (2H, s, OH), 7.68 (1H, d, J=7.9 Hz), 7.57 (1H, m, H-6), 7.39 (1H, s, H-4), 7.26 (1H, d, J=7.9 Hz, H-7), 7.07 (1H, s, H-2), 2.32 (3H, s, CH_3). ^{13}C-NMR (DMSO-d_6) δ : 161.5 (C1), 119.5 (C2), 149.3 (C3), 124.6 (C4), 137.5 (C5), 120.7 (C6) 161.7 (C7), 191.8 (C8), 181.7 (C9), 133.5 (C10), 113.5 (C4a), 116.0 (C8a), 133.2 (C9a), 21.0 (–CH_3).

Chrysophanol 8-*o*-β-D-Glucopyranoside (2) Yellow

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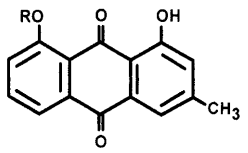
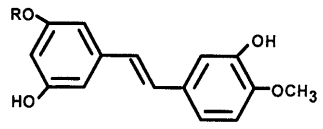


R : H - Chrysophanol (1)
D-glucose - Chrysophanol 8-O-β-D-glucopyranoside (2)



R : H - Rhapontigenin
D-glucose - Rhaponticin (3)

Fig. 1. Structure of Compounds Isolated from Rhei Undulati Rhizoma

needles. mp 259—260 °C, IR ν_{max}^{KBr} cm^{-1} : 3385 (OH), 2920 (C–H), 1671 (free C=O), 1632 (chelated C=O), 1594, 1448 (aromatic C=C), 1076 (sugar C–O). EI-Mass (*m/z*) : 416 [M^+], 254 [M^+−Glc]. ^{1}H-NMR (DMSO-d_6) δ : 12.97 (1H, s, OH), 7.87 (1H, d, J=7.4 Hz, H-5), 7.72 (1H, m, H-6), 7.53 (1H, d, J=7.4 Hz, H-7), 7.39 (1H, s, H-4), 7.21 (1H, s, H-2), 5.17 (1H, d, J=8.0 Hz, anomeric H), 2.43 (3H, s, CH_3), ^{13}C-NMR (DMSO-d_6) δ : 161.8 (C1), 119.5 (C2), 147.8 (C3), 122.7 (C4), 124.2 (C5), 136.1 (C6), 121.5 (C7), 158.4 (C8), 187.9 (C9), 182.3 (C10), 182.3 (C4a), 118.5 (C8a), 114.9 (C9a), 134.9 (C10a), 100,7 (C1′), 73.5(C2′), 76.7(C3'), 69.7(C4′), 77.5 (C5), 59.8 (C6), 20.8 (–CH_3)

Rhaponticin (3) White needles. mp 246—248 °C, IR ν_{max}^{KBr} cm^{-1} : 3482, 3341 (OH), 1612, 1583, 1513 (aromatic C=C). EI-Mass (*m/z*) : 420 [M^+], 258 [M^+-Glc]. 1H-NMR (DMSO-d_6) δ : 9.45 (1H, s, OH), 8.98 (1H, s, OH), 6.91, 6.83 (each H, d, J=16 Hz, olefinic H), 7.02 (1H, s, H-6), 6.58 (1H, s, H-2), 6.34 (1H, s, H-4), 4.80 (1H, d, J=6.5 Hz, anomeric H), 3.58 (3H, s, OCH3). 13C-NMR (DMSO-d_6) δ : 140.7 (C1), 106.4 (C2), 159.6 (C3), 104.1 (C4), 160.2 (C5), 108.3 (C6), 131.5 (C1′), 113.9 (C2′), 147.7 (C3′), 148.9 (C4′), 113.0 (C5′), 119.9 (C6′), 127.4 (Cα), 129.8 (Cβ), 102.0 (C1″), 74.6 (C2″), 77.8 (C3″), 71.1 (C4″), 77.8 (C5″), 62.1 (C6″), 56.5 (-OCH_3)

Isolation of Metabolites of Rhaponticin and Chrysophanol 8-*o*-β-D-Glucopyranoside by Human Intestinal Bacteria To obtain the metabolites of rhaponticin and chrysophanol 8-*o*-β-D-glucopyranoside by human intestinal bacteria, the reaction mixture contained 20 mg/ml of each compound and 5 g fresh human feces in a final volume of 100 ml of anaerobic dilution medium. The mixture was incubated at 37 °C for 20 h and was extracted three times with ethylacetate. The EtOAc-soluble portion of each reaction mixture was dried on a rotary evaporator under reduced pressure and subjected to silica gel column chromatography (2.5 ×15 cm) with $CHCl_3$–MeOH (10 : 1→10 : 4). Rhapontigenin and chrysophanol were obtained from each compound and were identified by comparison of the physico-chemical data in the literature.[11]

Rhapontigenin White needles. mp 184—188 °C, IR ν_{max}^{KBr} cm^{-1} : 3300, 1615, 1590, 1510. EI-Mass (*m/z*) : 258 [M^+].

Screening of Intestinal Bacterial Strains for Ability to Metabolize Rhaponticin and Chrysophanol 8-*o*-β-D-Glucopyranoside Each strain of precultured bacteria, which was isolated from human intestinal bacteria, was inoculated to GAM broth and cultured for 24 h at 37 °C under anaerobic conditions. Raphonticin or chrysophanol 8-*o*-β-D-glucopyranoside was incubated at 37 °C for 60 min with the cultured bacteria (wet weight, 5 mg). The reaction mixture was extracted twice with ethylacetate, and the ethylacetate fraction was analyzed by TLC.

Assay of Hyaluronidase Activity Hyaluronidase activity was determined by the modified Morgan–Elson method.[12]

Assay of β-Glucosidase Activities The enzyme mixture (total volume of 0.5 ml) contained 0.2 ml of 2 mM substrate, 0.2 ml of 0.2 M phosphate buffer, pH 7.0, and 100 μl of the cultured bacteria suspension (wet weight, 4 mg). The assay mixture was incubated at 37 °C for 60 min. The reaction was stopped by the addition of 0.2 ml of 1 N HCl, extracted with 2 ml ethylacetate and the amount of the metabolites was assayed by TLC. When *p*-nitrophenyl β-D-glucopyranoside was used as a substrate, the enzyme activity was measured according to the previous method.[13]

Thin-Layer Chromatography TLC for rhaponticin, rhapontigenin, chrysophanol 8-*o*-β-D-glycopyranoside and chrysophanol was performed on silica-gel plates (Merck, Silica-gel 60F_{254}) with $CHCl_3$–MeOH (4 : 1). The chromatograms of these compounds were quantitatively assayed with a TLC scanner.

Animals Male Hartley guinea pigs, male SD rats and male ICR mice were supplied from Daehan experimental animal breeding center.All animals were housed in wire cages, fed with usual laboratory chow (Samyang feed production Co.) and water *ad libitum*.

Assay of Histamine Release from Rat Peritoneal Exudate Cells Induced by Compound 48/80 or Calcium Ionophore A-23187 Male Wister rats (200±20 g) were exsanguinated and injected intraperitoneally with 20 ml of physiological solution (137 mM NaCl, 2.7 mM $CaCl_2$, 1.0 mM $MgCl_2 \cdot 6H_2O$, 5.6 mM glucose, 1 unit of heparin/ml and 5 mM phosphate buffer, pH 7.2). The abdominal region was gently massaged for 2 min and then the peritoneal exudate was collected. The cell suspension was centrifuged at 300×***g*** at 4 °C for 5 min and washed several times with physiological solution. The cells obtained from 5 animals were then pooled (5×10^4/ml cells).

The peritoneal exudate cell suspension (2.5 ml) prepared was mixed with various concentrations of test substances in 0.5 ml of each physiological solution and the mixture was preincubated at 37 °C for 5 min. The control and blank were treated in the same manner. The reaction mixture (3.0 ml) was then mixed with 0.5 ml of compound 48/80 (calcium ionophore A-23187 or egg albumin antigen). The mixture (3.5 ml) was cooled at 4 °C and centrifuged at 2500×***g*** at 4 °C for 10 min, and the histamine released in the supernatant and residue was measured according to the method of Shore *et al.*[14]

PCA Reaction An IgE-dependent cutaneous reaction was measured according to the previous method of Katayama *et al.*[15] The male ICR mice (25—30 g) were injected intradermally with 10 μg of anti-DNP IgE into each of four dorsal

skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hours later each mouse received an injection of 200 μl of 0.25% Evans blue PBS containing 100 μg of DNP-HSA *via* the tail vein. Rhaponticin, rhapontigenin or DSCG was administered 1 h prior to DNP-HSA injection. Thirty min after DNP-HSA injection, the mice were sacrificed and their dorsal skins were removed for measurement of the pigment area. After extraction with 1 ml of 1.0 N KOH and 9 ml of a mixture of acetone and 0.6 N phosphoric acid (13 : 5), the amount of dye was determined colorimetrically (the absorbance at 620 nm).

Anti-histamine Actions Male Hartley guinea pigs (300 $\pm$30 g) were sacrified by exsanguination and the ilea were prepared in cold Tyrode solution. The ileal strip prepared was then suspended in a 10 ml magnus tube (32 °C, 95% O_2+5% CO_2) containing the Tyrode solution. Each test drug was added to the preparation 30 s before the treatment of histamine (1×10^{-6} M). The percent contraction was shown as a percent of the maximal response to histamine.

RESULTS

Metabolism of Rhaponticin and Chrysophanol 8-*o*-β-D-Glucopyranoside from the Rhizome of *R. undulatum* by Human Intestinal Bacteria During the screening program to discover such compounds from natural products, the rhizome of *R. undulatum* was found to show inhibitory activity for the activation of hyaluronidase. From the hyaluronidase-inhibitory fraction of the rhizomes of *R. undulatum*, we isolated three compounds, chrysophanol, rhaponticin and chrysophanol 8-*o*-β-D-glucopyranoside. To investigate the relationship between the metabolism of rhaponticin and chrysophanol 8-*o*-β-D-glucopyranoside by human intestinal bacteria and their anti-allergic activity, these compounds were anaerobically incubated for 12 h with human fecal suspension.Then the metabolites were extracted with ethylacetate and analyzed by TLC. The metabolites of rhaponticin and chrysophanol 8-*o*-β-D-glucopyranoside were rapontigenin and chrysophanol by TLC and instrumental analysis (^{1}H-NMR, ^{13}C-NMR and EI-MS), respectively. To understand the metabolism of rhaponticin and chrysophanol 8-*o*-β-D-glucopyranoside, we screened intestinal bacterial strains for their ability to metabolize these compounds. Most strains of human intestinal bacteria transformed these compounds to their aglycones, respectively. The β-glucosidase activity of these representative intestinal bacteria was measured (Table 1). *Eubacterium* A-44, *Bifidobacterium breve* K-110 and *Bacteroides* JY-6 had the most potent β-glucosidase activity. These bacteria also potently transformed rhaponticin and chrysophanol 8-*o*-β-D-glucopyranoside to their aglycones, respectively.

Inhibition of Rhaponticin, Chrysophanol 8-*O*-β-D-Glucopyranoside and Their Metabolites on Hyaluronidase The inhibitory effects of Rhei Rhizoma and some products from it on the activation of hyaluronidase were examined (Table 2). Rhei Rhizoma had an inhibitory activity on the activation of hyaluronidase. Among the compounds isolated from Rhei Rhizoma, chrysophanol was found to weakly inhibit the activation of hyaluronidase. However, rhapontigenin, which was a metabolite by human intestinal bacteria, had dose-dependently the most potent inhibitory activity on the activation of hyaluronidase. The inhibitory potency of rhapontigenin was stronger than that of DSCG, which has been used as an anti-histamine drug. Its IC_{50} was 0.14 mM. The other glycosides did not inhibit the hyaluronidase.

Table 1. Activity Concerting Rhaponticin and Chrysophanol 8-*o*-β-D-Glucopyranoside to Their Aglycones by Defined Bacterial Strains from Human Feces

Microbe	Activity (μmol/h/mg wet weight)		
	PNG[a]	Rhaponticin	CG[b]
Fresh feces	0.027	0.025	0.031
Bacteroides JY-6	0.083	0.036	0.041
Bifidobacterium breve K-110	0.079	0.036	0.038
Eubacterium A-44	0.068	0.030	0.055
Bifidobacterium bifidum JCM 1254	0.073	0.032	0.022
Bacteroides stercoris HJ-15	0.081	0.029	0.016
E. coli HGU-3	0	0	0
Bifidobacterium B9	0.002	0	0
Lactobacillus L-2	0.019	0.015	0
Eubacterium L-8	0.033	0.001	0.001
Streptococcus LJ-22	0.052	0.032	0

a) PNG, *p*-nitrophenyl β-D-glucopyranoside. *b*) CG, chrysophanol 8-*o*-β-D-glucopyranoside.

Table 2. Inhibitory Activity of Certain Compounds from Rhei Undulati Rhizoma on Hyaluronidase

Agent	IC_{50} (mg/ml)
Rhaponticin	>2
Rhapontigenin	0.036 (0.14 mM)
Chrysophanol-Glc[a]	>2
Chrysophanol	1.8 (7.1 mM)
DSCG[b]	7.8 (15.2 mM)
Rhei Undulati Rhizoma	1.37

a) Chrysophanol-Glc, chrysophanol 8-*o*-β-D-glucopyranoside. *b*) DSCG, disodium cromoglycate.

Table 3. Inhibitory Effect of Certain Compounds and DSCG on Histamine Release from Rat Peritoneal Exudate Cells Induced by Compound 48/80 and Calcium Ionophore A-23187

Agent	IC_{50} (mM)	
	Compound 48/80	Calcium ionophore A-23187
Rhaponticin	0.29×10^{-3}	0.90×10^{-3}
Rhapontigenin	0.79×10^{-7}	0.25×10^{-6}
Chrysophanol 8-*o*-β-D-glucopyranoside	0.13×10^{-1}	0.56×10^{-2}
Chrysophanol	0.14×10^{-2}	0.39×10^{-2}
DSCG	0.32×10^{-2}	0.6×10^{-2}
Rhei Undulati Rhizoma	0.15×10^{-3} mg/ml	0.14×10^{-2} mg/ml

Histamine release (%) induced by compound 48/80 and calcium ionophore A-23187 was 82.7$\pm$4.4% and 75.0$\pm$8.8%, respectively. All values are means$\pm$S.D. (n=3).

Inhibition of Rhaponticin, Chrysophanol 8-*o*-β-D-Glucopyranoside and Their Metabolites on Histamine Release from Rat Peritoneal Mast Cells and PCA To learn whether the hyaluronidase-inhibitory rhapontigenin possesses anti-allergic activity, we then examined the inhibitory effects of natural products on the histamine release from rat peritoneal exudate cells induced by compound 48/80 and calcium ionophore A-23187 (Table 3). Among the compounds tested, rhapontigenin had the strongest inhibitory activity on

Table 4. Inhibitory Effect of Certain Compounds and DSCG on PCA Reaction

Agent	Dose (mg/kg)	Route	Inhibition (%)
Control (saline)	—	*p.o.*	—
Rhaponticin[a)]	100	*p.o.*	53±2
	100	i.p.	18±3
Rhapontigenin	25	i.p.	48±9
	50	i.p.	85±4
DSCG	100	*p.o.*	38±2

All agents were administered *p.o.* or i.p. prior to challege with antigen. Each experiment consisted of 5 observations. *a*) If 100 mg of rhaponticin was metabolized by intestinal bacteria, 61.4 mg of rhapontigenin could be produced.

the histamine release induced by compound 48/80. In this case, the inhibitory effect of chrysophanol was stronger than that of DSDG. However, glycosides of these compounds, rhaponticin and chrysophanol 8-*o*-β-D-glycoside, exhibited very little inhibitory activity. These results suggest that the potent inhibitory compounds on the activation of hyaluronidase may be the anti-histamine drugs. Furthermore, rhapontigenin and chrysophanol inhibited histamine release from rat peritoneal exudate cells induced by calcium ionophore A-23187 in a dose related manner. DSCG weakly inhibited histamine release from rat peritoneal exudate cells induced by calcium ionophore A-23187. However, rhapontigenin and chrysophanol potently inhibited histamine release induced by a high concentration of compound 48/80 and calcium ionophore A-23187.

To determine the inhibitory effect of rhaponticin and its metabolite on PCA reaction in mice, these compounds were administered orally or intraperitoneally 60 min prior to challenge with antigen (Table 4). These compounds all inhibited PCA reaction on rats. Among the substances tested, rhapontigenin orally administered showed the strongest inhibitory activity and significantly inhibited PCA at doses of 25 and 50 mg/kg with inhibitory activity of 48 and 85%, respectively. The inhibitory activity of i.p. treated rhapontigenin was stronger than those of i.p. and *p.o.* treated rhaponticin and DSCG. while the inhibitory activity of *p.o.* treated rhaponticin was stronger than those of i.p. treated rhaponticin and DSCG.

DISCUSSION

DSCG and tranilast are both anti-allergic drugs and were made from natural products. These drugs are strong inhibitors of hyaluronidase, whereas compound 48/80, a histamine releaser, is an activator of hyaluronidase. These facts suggest that the anti-hyaluronidase activity of some allergic drugs must be related to the mechanism of inhibitory activity on histamine releaser acting on mast cells. Furthermore, if strong hyaluronidase-inhibitory substances are discovered, they can be lead compounds for developing new anti-allergic drugs.

In the present study, we investigated the inhibitory activity of rhaponticin, chrysophanol 8-*o*-β-D-glucopyranoside and their metabolites on the activation of hyaluronidase. Our results showed that these compounds potently inhibited the activation of hyaluronidase dose-dependently. Among the substances tested, rhapontigenin showed the strongest inhibitory activity on hyaluronidase as well as histamine release from rat peritoneal mast cells. These results suggest that their inhibition might be due to protection of the cytolytic response by compound 48/80 and calcium ionophore A-23187 as well as inhibition of hyaluronidase. The histamine release from mast cells is an essential step in the pathological process of type I allergy. These results suggest that rhapontigenin and chrysophanol may possess cell membrane stabilizing activity. Futhermore, when we determined the Schultz–Dale reaction using sensitized quinea pig ileum and anti-histamine action using guinea pig ileum, rhapontigenin had potent inhibitory activity on Schultz–Dale reaction at 1×10^{-5} M (data not shown); however, it did not cause a significant anti-histamine action. These results suggest that the inhibitory activity of rhapontigenin on Schultz–Dale reaction was not relevant to its anti-histamine action. This rhapontigenin had the most potent inhibitory activity on PCA reaction, and the inhibitory activity of *p.o.* treated rhaponticin was stronger than that of i.p. treated rhaponticin. These results suggest that, when rhaponticin was orally treated, it was easily metabolized to rhapontigenin and the metabolite was responsible for the inhibitory activity on PCA reaction.

In this study, rhapontigenin, the metabolite of rhaponticin by human intestinal bacteria, demonstrated an inhibitory activity on the activation of hyaluronidase, a histamine release from mast cell and PCA reaction. Finally, we believe that rhaponticin in the rhizome of *R. undulatum* is a prodrug that has extensive anti-allergic properties.

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