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Biotransformation of Glycyrrhizin by Human Intestinal Bacteria and its Relation to Biological Activities

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The relationship between the metabolites of glycyrrhizin (18β-glycyrrhetinic acid-3-O-β-D-glucuronopyranosyl-(1→2)-β-D-glucuronide, GL) and their biological activities was investigated. By human intestinal microflora, GL was metabolized to 18β-glycyrrhetinic acid (GA) as a main product and to 18β-glycyrrhetinic acid-3-O-β-D-glucuronide (GAMG) as a minor product. The former reaction was catalyzed by *Eubacterium* L-8 and the latter was by *Streptococcus* LJ-22. Among GL and its metabolites, GA and GAMG had more potent *in vitro* anti-platelet aggregation activity than GL. GA also showed the most potent cytotoxicity against tumor cell lines and the potent inhibitory activity on rotavirus infection as well as growth of *Helicobacter pylori*. GAMG, the minor metabolite of GL, was the sweetest.

Key words: Glycyrrhizin, β-glucuronidase, Intestinal bacteria, *Eubacterium* L-8, Anti-rotavirus activity, Anti-cytotoxic activity

INTRODUCTION

Most herbal medicines are orally administered as decoctions. Therefore, the components of these herbal medicines are inevitably brought into contact with intestinal microflora in the alimentary tract. Most components may be transformed by the intestinal bacteria before their absorption from the gastrointestinal tract. Studies on the metabolism of the components of herbal medicines by human intestinal microflora are of a great importance in understanding of their biological effects (Kim, 1995; Kobashi and Akao, 1997).

Glycyrrhizin (18β-glycyrrhetinic acid-3-O-β-D-glucuronopyranosyl-(1→2)-β-D-glucuronide, GL), which is a main component of licorice extract (*Glycyrrhiza glabra*), is ingested orally as a sweetener as well as a component in Oriental medicine. GL has steroid-like action, antiviral activity and anti-inflammatory activity (Kumagai *et al.*, 1957; Pompeo *et al.*, 1979; Abe *et al.*, 1982). By the oral administration of GL to human, 18β-glycyrrhetinic acid (GA) was detected in the sera, but GL was not (Nakano *et al.*, 1980). Hattori *et al.* (1983) reported that GL was transformed to GA by human intestinal bacteria. These results suggest that even if GL is orally administered to human, GL is converted to GA by human intestinal bacteria before GL is absorbed into the body. Akao *et al.* (1991) reported that 18β-glycyrrhetinic acid-3-O-β-D-glucuronide (GAMG) as well as GL were metabolized by the β-glucuronidase of human intestinal bacteria. We reported that GAMG as a minor metabolite and GA as a main metabolite were isolated, when GL was incubated with human intestinal microflora (Kim *et al.*, 1996; Kim *et al.*, 1999). We proposed two pathway in relation to the metabolism of GL to GA by human intestinal bacteria: one is the main pathway which directly metabolizes GL to GA and the other is the minor pathway which metabolizes GL to GA via GAMG.

However, the studies on biological activities of the metabolites of GL in connection with the metabolism of GL were not complete. Here we isolated the intestinal bacteria directly metabolizing GL to GA from human intestinal microflora, which is the unknown bacterium for the metabolism of GL. Futhermore, to understand the relationship between the metabolites of GL and their biological activity, we investigated *in vitro* some biological activities of the metabolites of GL.

MATERIALS AND METHODS

Materials and microorganisms

Bovine and *E. coli* β-glucuronidases, *p*-nitrophenyl β-D-

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glucuronide, GA and GL were purchased from Sigma Chem. Co. (U.S.A). General anaerobic medium (GAM) and glucose blood liver broth (BL) were purchased from Nissui Pharm. Co., Ltd. and other media were from Difco Co. (U.S.A). GAMG was prepared according to our previous method (Kim *et al.*, 1999). HP ATCC43504 was purchased from ATCC. HP NCTC11638 was from NCTC. Other HP (HP 82548) was a clinical isolate selected from Korean gastroscopic sample. Wa virus (Rotavirus) was kindly donated from Japan NIH. *Streptococcus* LJ-22 was isolated in our previous study (Kim *et al.*, 1999).

Isolation of intestinal bacteria metabolizing GL

A suspension of the fresh feces of a Korean man was diluted 10^5- to 10^8-fold with GAM medium. An aliquot (200 µl) of the 10^7-diluted human feces was inoculated onto a BL agar plate, which was anaerobically incubated at 37°C for 4 days. Each colony was cultured in 10 ml of GAM broth containing 0.1% GL. The cultured medium was extracted with 10 ml of ethylacetate. After evaporating the ethylacetate fraction to dryness, the residue was analyzed for GL, GAMG and GA by TLC. L-8 was isolated as a bacterium transforming GL to GA. Identification of the isolated bacterium, L-8, was performed according to Bergey's manual (Moore and Moore, 1984). L-8 was *Eubacterium spp.* (Table I).

Biotransformation of GL, GAMG and p-nitrophenyl β-D-glucuronide by glucuronidases of human intestinal bacteria

GL-metabolizing activity was measured as follows. The assay mixture contained 0.2 ml of 2 mM GL (or other substrates) and 0.2 ml of the enzyme in a final volume of 1 ml of 0.1 M phosphate buffer, pH 7.0. The mixture was incubated at 37°C for 2 h and was extracted twice with 5 ml of ethylacetate. The ethylacetate fraction was analyzed by TLC. The quantity of these compounds was determined with a TLC scanner (Shimazu CS-9301). When *p*-nitrophenyl β-D-glucuronide was used as a substrate, the activity was assayed according to the previous method (Kim *et al.*, 1999).

TLC

TLC for GL, GAMG and GA was performed on silica-gel plates (Merck, silica gel 60F-254). The developing solvents for determining GA were $CHCl_3$/petroleum ether/AcOH (6:6:1) and those for determining GAMG and GL was BuOH/$CHCl_3$/AcOH/H_2O (4:1:4:1).

Isolation of GA transformed from GL by β-glucuronidase of *Eubacterium* L-8

Eubacterium L-8, which has been isolated from human intestinal bacteria, was cultured in 5 L tryptic soy (TS) broth with 1 g of GL. After cultivation at 37°C for 24 h, it was adjusted to pH 2 with 1 N HCl and extracted twice with ethylacetate. After evaporating the ethylacetate fraction to dryness, the resulting powder was applied to silica gel column chromatography (2.5 × 40 cm) using $CHCl_3$/methanol (4:1) as the elution solvent. The isolated GA was crystallized with $CHCl_3$/MeOH (5:1).

^{13}C-NMR(125 MHz, $CDCl_3$) - 40.21(C-1), 27.61(C-2), 76.99 (C-3), 40.53(C-4), 56.43(C-5), 18.44(C-6), 33.82 (C-7), 44.64 (C-8), 63.13(C-9), 38.08(C-10), 202.65(C-11), 128.96(C-12), 172.77(C-13), 46.76(C-14), 26.97(C-15), 27.41(C-16), 32.98 (C-17), 49.93(C-18), 42.43(C-19), 44.90(C-20), 32.02(C-21), 39.02(C-22), 28.41(C-23), 16.94(C-24), 16.96(C-25), 19.31 (C-26), 23.81(C-27), 28.75(C-28), 29.19(C-29), 180.38 (C-30).

Preparation of β-glucuronidase from *Streptococcus* LJ-22 and *Eubacterium* L-8

Streptococcus LJ-22 was cultured in 5 L of TS broth and partially purified according to our previous method (Kim *et al.*, 1999). The specific activity of the partially purified enzyme was 0.6 nmol/min/mg protein (substrate, GL). *Eubacterium* L-8 was cultured in 10 L of TS broth, respectively. They washed with saline, sonicated, centrifuged and fractionated with ammonium sulfate (35-70% saturation). The resulting precipitate was dissolved in 30 ml of 20 mM phosphate buffer (pH 7.0), dialyzed with the same buffer, and the inner dialysate was applied to a column of QAE (2.7 × 5 cm) previously equilibrated with 0.05 M phosphate buffer. The column was then washed with 200 ml of the same buffer and eluted with a linear gradient between 100 ml of the same buffer and 100 ml of 50 mM phosphate buffer containing 1 M KCl. Fractions (5 ml) were collected and monitored by measuring the enzyme activity and the absorbance at 280 nm. The active fractions were pooled and concentrated by ultrafiltration through a UM-10 membrane (Amicon Corp., U.S.A.). The concentrate was dialyzed with the 50 mM phosphate buffer. The specific activity of the partially purified enzyme was 0.3 nmol/min/mg protein (substrate, GL).

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Assay of *in vitro* cytotoxicity

The *in vitro* cytotoxicity was tested against SNU C4 (human colon cancer cell line), P388 (mouse lymphatic neoplasma cell line), L1210 (mouse lymphocytic leukemia cell line) and MA104 (Macacus' rhesus monkey kidney cell line) by MTT [3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay according to the method of Carmichael *et al.* (1987). Each cultured cell line was harvested, counted, and inoculated at the appropriate concentrations (180 µl volume: 4×10^4 cells/ well for P388 and L1210; 3×10^4 cells/ well for SNU C4 and MA104) into 96-well microtiter plates. And then P338

and L1210 cell lines were cultured for 2 h and SNU C4 and MA104 cell lines were for 24 h. These cells were exposed to the test compounds for 2 days at 37°C. 50 μl MTT solution (2 mg/ml in PBS) was added to each well and the plates were incubated for 4 h. After aspiration of the medium, DMSO (100 μl) was added to solubilize the MTT-formazan product. The plate was read on a microplate reader on a 540 nm. The 50% inhibitory concentration (IC_{50}) of tumor cell growth was defined compared with the control cell culture.

Assay of anti-platelet aggregation activity

The anti-platelet aggregation activity was determined according to smearing of Yun-Choi *et al.* (1985). Platelet rich plasma (PRP) was prepared by centrifugation of the citrated blood at 200 × g for 10 min and platelet poor plasma (PPP) was obtained from the residue by centrifugation at 900xg for 30 min. Platelet number was adjusted to 3×10^{11}/L by mixing PRP and PPP. PRP (20 μl) and 10 μl of 4 mM GL (or its metabolites) were incubated at 37°C for 8 min and then 10 μl of 1 mM ADP (1 mg/ml) was added to induce platelet aggregation. The reaction mixture was smeared into a slide glass, then stained with Wright-Giemsa and measured with a microscope. The degree of aggregation was graded as described: -, no aggrega-tion as shown with PRP plus saline alone (negative control); ±, slight aggregation of platelets; +, less aggregation than with PRP plus saline and an appropriate aggregating agent (ADP); ++, as much aggregation as with PRP plus saline and an appropriate aggregating agent (positive control).

Assay of HP growth inhibition and HP urease

HPs were inoculated into brucella agar plates supplemented with 7% horse serum and cultured for 3 days at 37°C in an anaerobic jar with AnaeroPak Campylo. Growth inhibition assay of HP was performed according to the previous method (Bae *et al.*, 1998). 1 ml of each compound was added into petri dish containing unsolidified 7 ml Brucella agar supplemented with 7% horse serum. Final concentrations of each isolated compound were 400, 200, 100, 50, 25, 12.5 and 6.25 μg/ml. And then the HP of approximately 5×10^5 cfu were inoculated into the agar plates and cultured microaerobically for 3 days at 37°C in an anaerobic jar (85% N_2, 10% CO_2, 5% O_2). The MIC was determined after an incubation period of 72 h. Ampicillin was used as a positive control. All experiments were conducted in triplicate.

Culture of rotavirus and assay of its inhibitory activity of GL and its metabolites

MA104 cell was cultured in DMEM containing 10% FBS, 1% antibiotics-antimycotics and 3.5 g/L sodium bicarbonate (CO_2 incubator, 37°C). For nonactivated virus, rhesus rotavirus was activated with 100 μg of trypsin per ml at 37°C for 1 h. Washed MA104 cell monolayers were inoculated with activated rotavirus at a multiplicity of infection of 1 to 5. After 1 h the inoculation was aspirated, the flasks were washed twice and refed with medium DMEM with trypsin, and the cells were grown until a cytopathic effect was visible, usually within 3-5 days. Quantifying the rotavirus in a given sample was performed according to end-point dilution assay. The assay of inhibitory activity of GL and its metabolites, was based on the inhibition of rotavirus-induced cytopathogenicity. Briefly, 50 μl of 10^{-3}-diluted Wa virus (1×10^3 pfu) was infected into 100 μl of MA104 cells (3×10^5 cells/ml) containing 50 μl of sample. And then the cells were grown until a cytopathic effect was visible and measured the inhibitory activity of sample on rotavirus infectivity (Song and Kim, 1998).

RESULTS AND DISCUSSION

Screening of the human intestinal bacteria transforming GL to GA

When GL was anaerobically incubated for 24 h with a bacterial mixture from human feces, we isolated two metabolites, GA and GAMG, as reported previously. GA was a main metabolite. We screened the bacteria metabolizing GL to GA from two hundreds of intestinal bacteria from human feces. We isolated the bacterium potently metabolizing GL to GA. This bacterium was different from the GL-metabolizing bacterium previously isolated from human intestinal microflora. L-8 was an anaerobic and gram-positive rod. The characteristics of L-8, are shown in Table I. L-8 was identified to be *Eubacterium spp.*

Characterization of partially purified β-glucuronidase from L-8

Production of β-glucuronidase was gradually increased together with the growth of *Eubacterium* L-8 and thereafter slowly decreased according to its growth. The enzyme was inducible: when *Eubacterium* L-8 was cultured in a medium containing GL, the enzyme was induced

Table I. Characteristics of *Eubacterium* L-8 isolated from human intestinal microflora

Characteristic	L-8	Characteristic	L-8
Gram stain	+	H_2S production	-
Shape	rod	Simmons citrate	-
Colony diameter	>0.5 mm	Gas production	+
Methyl red	+	Catalase	-
Voges-Proskauer	-	Urease	-
Nitrate reduction	-	β-glucuronidase	+

Table II. Substrate specificity of glucuronidases from intestinal bacteria and mammalian tissues

Enzyme	Activity (nmol/min/mg protein)			
	PNGu[a]	GL→GAMG	GL→GA	GAMG→GA
Eubacterium L-8 β-glucuronidase	13.6	0	0.3	0.8
Streptococcus LJ-22 β-glucuronidase	0.02	0.6	0	0
E. coli HGU-3 β-glucuronidase	48	0	0	0.01
E. coli β-glucuronidase (commercial)	1840	0	0	29.3
Bacteroides J-37 glucuronidase	2.7	0	1	107

[a] p-Nitrophenyl β-glucuronide.

more than 2-fold according to the increase of GL concentration. *Eubacterium* L-8 was cultured anaerobically for 10 h at 37°C in TS broth. The enzyme was then partially purified 8-fold compared to a crude extract by ammonium sulfate fractionation and QAE-cellulose. The optimal pH of the partially purified enzyme was 6.5 (Data not shown).

To understand the characterization of the partially purified β-glucuronidase, the glycyrrhizin metabolizing activities of many putative glucuronidases originating from some intestinal bacteria were investigated (Table II). Glcyrrhizin metabolizing activities of these enzymes were quite different. Commercial *E. coli* β-glucuronidase and *E. coli* HGU-3 β-glucuronidase weakly hydrolyzed GAMG to GA, but did not hydrolyze GL. The glucuronidase isolated from *Bacteroides* J-37 hydrolyzed both GAMG and GL to GA (Kim *et al.*, 1987). The Glcyrrhizin metabolizing activity of the putative glucuronidase isolated from *Bacteroides* J-37 was different from that of the above β-glucuronidase. Akao *et al.* (1987) also reported that *Eubacterium sp.* GLH β-glucuronidase hydrolyzed GL and synthesized β-GAMG. The putative *Streptococcus* LJ-22 β-glucuronidase hydrolyzed GL to GAMG. However, it did not hydrolyze GAMG or GL to GA. Here, the putative β-glucuronidase partially purified from *Eubacterium* L-8 could hydrolyze GL to GA. This β-glucuronidase proportionally hydrolyzed GL to GA with the lapse of incubation time (Fig. 1). Futhermore, this β-glucuronidase hydrolyzed GAMG as well as GL to GA (not GAMG). The glycyrrhizin transforming activity of this enzyme was similar to that of the enzyme reported by Akao *et al.* (1987). These results suggest that the metabolic procession of GL to GA by human intestinal bacteria followed two pathways: one was the main pathway directly metabolizing GL to GA and the other was the minor pathway metabolizing GL to GA *via* GAMG (Fig. 2).

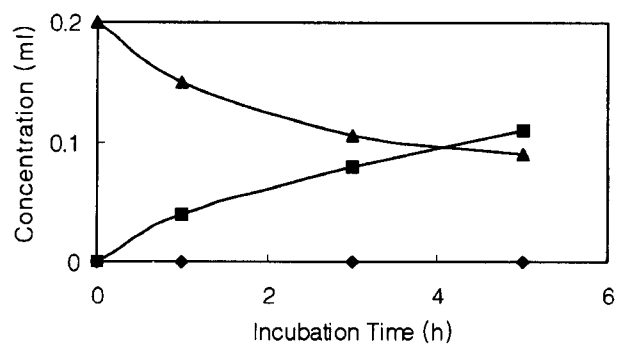

Fig. 1. Time course of reaction mixture by *Eubacterium* L-8 β-glucuronidase Symbols indicate the following: ◆, GL; ■, GAMG; ▲, GA.

Biological activity of GL and its metabolites

The anti-platelet aggregation activity, cytotoxic activity against tumor cell lines, anti-HP activity and anti-Rotavirus activity of the metabolites of GL were investigated. Their anti-platelet aggregation activity was shown in Table III. GAMG and GA had more potent anti-platelet aggregation activity than GL. *In vitro* cytotoxic activity of glycyrrhizin and its metabolites on tumor cell lines was shown in Table IV. GA showed the most potent cytotoxicity against tumor cell lines. The ED_{50} values of GA were 0.1-0.45

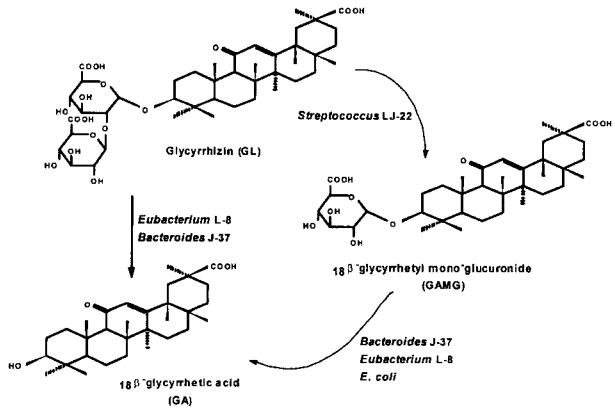

Fig. 2. Proposed metabolic pathway of glycyrrhizin by human intestinal bacteria

Table III. Antipletelet aggregation activity of glycyrrhizin and its metabolites

Compound	Platelet aggregation activity
Positive control	++
Negative control	-
Aspirin	-
Glycyrrhizin	+
GAMG	-
GA	-

Table IV. *In vitro* cytotoxicity of glycyrrhizin and its metabolites against tumor cell lines

Compound	IC_{50} (mM)						
	SNU C4	SNU-1	P-388	L-1210	HepG2	A549	MA104
Glycyrrhzin	>1	>1	>1	1	>1	>1	>1
GAMG	>1	>1	>1	1	>1	>1	>1
GA	0.4	0.45	0.1	0.1	0.1	0.1	1
adriamycin	0.05	0.001	0.003	0.002	>1	0.1	1

Table V. Anti-*Helicobacter pylori* activity of glycyrrhizin and its metabolites

Compound	MIC (mg/ml)		
	HP ATCC43504	HP NCTC11638	HP 82548
Glycyrrhizin	>0.4	>0.4	>0.4
GAMG	>0.4	>0.4	>0.4
GA	0.2	0.2	0.2

Table VI. Inhibitory effect of glycyrrhizin and its metabolites on rotavirus infection

Compound	IC_{50} (μg/ml)
Glycyrrhizin	0.05 ± 0.018
GAMG	0.10 ± 0.021
GA	0.01 ± 0.003

mM. However, GL and GAMG were weak. Anti-HP activity of GL and its metabolites was shown in Table V. GA showed the most potent inhibitory activity on the HP growth. The MIC values of GA were 0.2 mg/ml. However, GL and GAMG had not the inhibitory activity of HP growth. Anti-rotavirus activity of GL and its metabolites was shown in Table VI. GA had the most potent activity on rotavirus infection. GL and GAMG had not the inhibitory activity on rotavirus infection. These results suggest that intestinal bacteria may play an important role in anti-platelet aggregation activity, cytotoxicity and anti-HP activity of GL. The relative sweetness compared to 6% sucrose was determined for an aqueous solution of each compound by panel of five professional tasters in the same manner as reported previously. The intensity of sweetness of GAMG was found to be extremely higher than that of GL. The intensity of sweetness of GAMG was 940-fold, compared to sucrose, although GL was 190-fold. This result suggest that GAMG could be useful as a new sweet food additive.

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