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Metabolism of Glycyrrhizin and Baicalin by Human Intestinal Bacteria

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By human intestinal bacteria, glycyrrhizin (18β-glycyrrhetic acid β-D-glucuronyl α-D-glucuronic acid, GL) and baicalin (baicalein β-D-glucuronic acid) were metabolized to glycyrrhetinic acid and baicalin, respectively. However, α-glucuronidase of *Bacteroides* JY-6 isolated from human intestinal bacteria hydrolyzed GL or 18β-glycyrrhetinic acid α-D-glucuronic acid to 18β-glycyrrhetic acid but did not baicalin. However, *E. coli* β-glucuronidase from human intestinal bacteria hydrolyzed baicalin to baicalein, but did not GL. β-Glucuronidase of mammalian tissues hydrolyzed both GL and baicalin.

Keyword : Glycyrrhizin, Intestinal bacteria, Baicalin, *Bacteroides* J-37

INTRODUCTION

Glycyrrhizin (18β-glycyrrhetic acid β-D-glucuronyl α-D-glucuronic acid, GL) which is a main component of liquore extract *(Glycyrrhiza glabra)*, and baicalin (baicalein β-D-glucuronide) which is a main component of Scutellariae Radix (*Scutellaria baicalensis*) are ingested orally as a component in the oriental medicine. GL has a steroid-like action, antiviral activity, anti-inflamatory activity and so on (Kumagai *et al.*, 1957; Pompeo *et al.*, 1979; Abe *et al.*, 1982; Tangari *et al.*, 1965). Baicalin also has an anti-inflammatory activity, antioxidant activity and so on (Khoda, 1987).

When GL was orally or intravenously administered on human, metabolites were different in the sera of human subject according to administered routes. By oral administration of GL, 18β-glycyrrhetic acid (GA), not GL, was detected in the sera (Nakano *et al.*, 1980; Sakiya *et al.*, 1979). By intravenous administration of GL, 18β-glycyrrhetic acid α-D-glucuronic acid (GAMG), GL and GA were detected in the sera. These GA and GAMG were produced by lysosomal β-glucuronidase of liver. This GAMG should be excreted into the bile and transformed to GA by intestinal bacteria. Hattori *et al.* (1983) reported that GL was converted to GA by human intestinal bacteria. Furthermore, Akao *et al.* (1991) reported that GL and GAMG were metabolized by β-glucuronidase of human intestinal bacteria. However, the glucuronic acid linkage of GAMG is different to that of baicalin : the former is α-form but the latter is β-form.

In order to illustrate the difference of the enzymatic reaction between these compounds, we studied the metabolism of baicalein and GL/GAMG by intestinal bacteria and liver.

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MATERIALS AND METHODS

Materials

E. coli and bovine β-glucuronidases, p-nitrophenyl β-D-glucuronide, glycyrrhetic acid, glycyrrhizin and baicalin were purchased from Sigma Chem. Co. (U.S. A.). β-Glucuronidase of *E. coli* HGU-3 isolated from human intestinal bacteria was purified according to our previous method (Kim *et al.*, 1995) and rat lysosomal β-glucuronidase was purified according to the method of Akao *et al.* (1991). J-37 was isolated from the fresh feces of the healthy Korean man.

Methods

Isolation of GAMG produced from GL by rat liver β-glucuronidase : GL was converted to GAMG by rat lysosomal β-glucuronidase purified according to the previous method (Akao *et al.*, 1991). The partially purified β-glucuronidase was incubated with 500 mg of GL in 100 ml of 0.1 M acetate buffer (pH 5.5). After inubation at 37°C for 24h, the reaction mixture was extracted twice with 500 ml ethylacetate. After evaporating the ethylacetate fraction, the resulting powder was applied to silica gel column chromatography (2.5×40 cm), using acetic acid/butanol/1,2-dichloroethane/H_2O (4 : 1 : 4 : 1) as the elution solvent.

The isolated GAMG was recrystallized with MeOH. GAMG had the following spectral properties; FAB-MS *m/z* (negative ion): 646 (M+), 469 (aglycone-1), 451 (469-H_2O).

^{1}H-NMR (500 MHz, $CDCl_3$) : 0.83 (3H, s), 0.87 (3H, s), 1.06 (3H, s), 1.13 (3H, s), 1.16 (3H, s), 1.41 (3H, s), 4.37 (1H, d, J=7.8), 5.57 (1H,s)

^{13}C-NMR (500 MHz, $CDCl_3$) : aglycone moiety- 40. 21 (C-1), 27.61 (C-2), 90.74 (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)

sugar moiety- 106.95 (C-1'), 77.71 (C-2'), 76.54 (C-3'), 75.31 (C-4'), 73.18 (C-5'), 172.70 (C-6')

Isolation of J-37 hydrolyzing GL to GA and LJ-27 hydrolyzing GL to GAMG : A suspension of human feces was diluted with GAM medium. An aliquot (10 ml) of the 10^7-diluted human feces was inoculated in the plate media containing GL. These inoculated plates were anaerobically incubated at 37°C for 3-5 days. Each colony from the plate was incubated in 10 ml of GAM broth containing GL (0.1 mg/ml). The cultured medium was extracted with 10 ml of ethylacetate. After evaporating the ethylacetate fraction, it was analyzed for GL, GAMG and GA by TLC. The bacteria producing GA or GAMG from GL were selected. Identification of the isolated bacteria was performed according to Bergey's mannual.

Preparation of glucuronidases from human intestinal bacteria and rat liver : J-37 were cultured in 1L of GAM broth, washed with saline, sonicated, centrifuged and fractionated with ammonium sulfate of 60% saturation, consecutively. The resulting precipitate was dissolved in 20 mM phosphate buffer (pH 7.0) and dialyzed against the same buffer. It was used as the crude enzyme.

Purification of β-glucuronidase from *E. coli* HGU-3 was performed according to our method (Kim *et al* 1995).

Fresh liver from rat (Wistar, male 200 g) was homogenized and the lysosomal fraction was isolated according to the method of Akao *et al* (1991). The lysosome fraction was suspended in 20 mM acetate buffer (pH 5.5), sonicated and then centrifuged at 100, 000×g for 90 min. The resulting supernatant was used as the crude β-glucuronidase of rat liver.

Thin layer chromatography : TLC for GL, GAMG and GA was performed on silica gel plates (Merck, silica gel 60F-254) as follows; developing solvent A system, $CHCl_3$/petroleum ether/acetic acid (5 : 5 : 1) for GA; B system, acetic acid/butanol/1,2-dichloroethane/H_2O (4 : 1 : 4 : 1) for GL and GAMG. The quantity of these compounds were assayed with a TLC scanner (Shimazu CS-920).

Time courses of metabolism of GL by glucuronidases of rat liver, bovine and human intestinal bacteria : GL-metabolizing activities were measured as follows. The assay mixture contained 10 ml of 2 mM GL (or GAMG) and 10 ml each enzyme solution (glucuronidases of rat liver, bovine or human intestinal bacteria) in a final volume of 50 ml of 0.1M phosphate buffer (pH 7.0). The mixture was incubated at 37°C for 2 days and an aliquot (5 ml) of the reaction mixture was periodically extracted twice with 5 mL of ethylacetate. The ethylacetate fraction was analyzed for GL, GAMG and GA by TLC.

RESULTS

Metabolites of GL and baicalin by human intestinal bacteria

To investigate the metabolite of GL by human intestinal bacteria, GL was anaerobically incubated for 32h with a bacterial mixture from human feces. And then the metabolites was extracted with ethylacetate and separated by means of column chromatography.

Two metabolites, main metabolite A (>90%) and minor metabolite B (<2%), were isolated.

Main metaoblite A, Rf=0.57 on TLC (deveolping solvent A system), was obtained as colorless prisms. Its mass spectrum showed a molecular ion at *m/z* 470 (M+), corresponding to molecular formula $C_{30}H_{46}O_4$. The proton and carbon-13 nuclear magnetic resonance spectra showed that the chromone nucleus was intact but the diglucuronyl moiety was missing. The spectroscopic data agreed with those of GA.

Minor metabolites B, Rf=0.6 on TLC (deveolping solvent B system), was obtained as colorless needles.

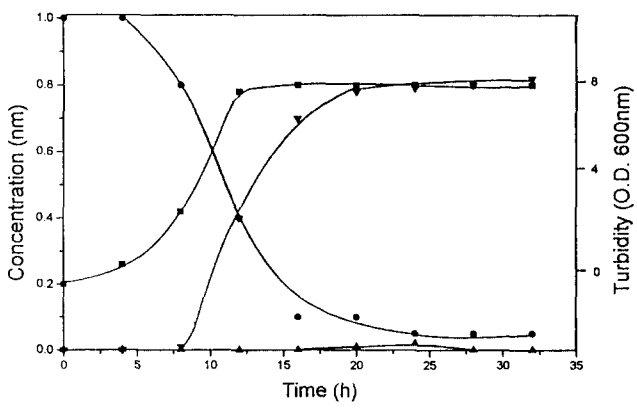


Fig. 1. Metabolic time course of glycyrrhizin by human intestinal bacteria. The incubated reaction mixture was acidified and extracted with ethylacetate. The upper phase was chromatographed on TLC plate and analyzed by TLC scanner. ●, glycryrrhizin; ▲, GAMG; ▼, GA; ■, turbidity

Its mass spectrum showed a molecular ion at *m/z* 646 (M+), corresponding to molecular formula $C_{36}H_{54}O_{10}$. The proton and carbon-13 nuclear magnetic resonance spectra showed that the chromone nucleus and monoglucuronyl moiety were intact but only one glucuronyl moiety was missing. The spectroscopic data agreed with those of GAMG.

The time course of the bacterial transformation of GL was shown in Fig. 1. GL started to be converted to GA 6h after incubation with the bacterial mixture and then most of GL was transformed to GA within 24h. However, GL was weakly converted to GAMG 24h after incubation.

To investigate the metabolite of baicalin by human intestinal bacteria, baicalin was anaerobically incubated for 30h with a bacterial mixture from human feces. And then the metabolite was extracted with ethylacetate and separated by means of column chromatography. The metabolite was found out as baicalein by the spectroscopic data of ^{1}H- and ^{3}C-NMR and FAB-MS.

Screening of GL-hydrolyzing bacteria from human intestinal bacteria

Two hundreds of human intestinal bacteria isolated from human feces were anaerobically cultured and assayed for GL-transforming activity. We isolated the bacterial strain, J-37, transforming GL to GA. The characteristics of the GL-transforming bacterium, J-37, was shown in Table I. J-37 was *Bacteroides spp.*, which was a gram-postive, gas-forming, indole-productive and catalase-negative rod. J-37 could not transform baicalin to baicalein. *E. coli* HGU-3, which was an human intestinal bacterium isolated by us, could transform baicalin to baicalein very well, but could not transform GL to GAMG. The bacteria transforming GL to GAMG is on screening.

Table I. Characteristics of Bacteroides J-37 isolated from human intestinal microflora

Characteristic	J-32	Characteristic	J-32
Shape	rod	H_2S production	-
Gram	-	Simmon's citrate	-
MR	-	Catalase	-
VP	-	Gas production	+
Nitrate reduction	-	β-Glucosidase	+
Indole production	-	α-Rhamnosidase	+

Metabolism of GL and baicalin by several kinds of glucosidases

The GL- and baicalin-hydrolyzing activity of several kinds of glucuronidases were investigated (Table II). GL, which has β-D-glucuronide and α-D-glucuronide on 18β-glycyrrhetic acid, GAMG, which has only α-D-glucuronic acid on 18β-glycyrrhetic acid, and baicalin, which has only β-D-glucuronide

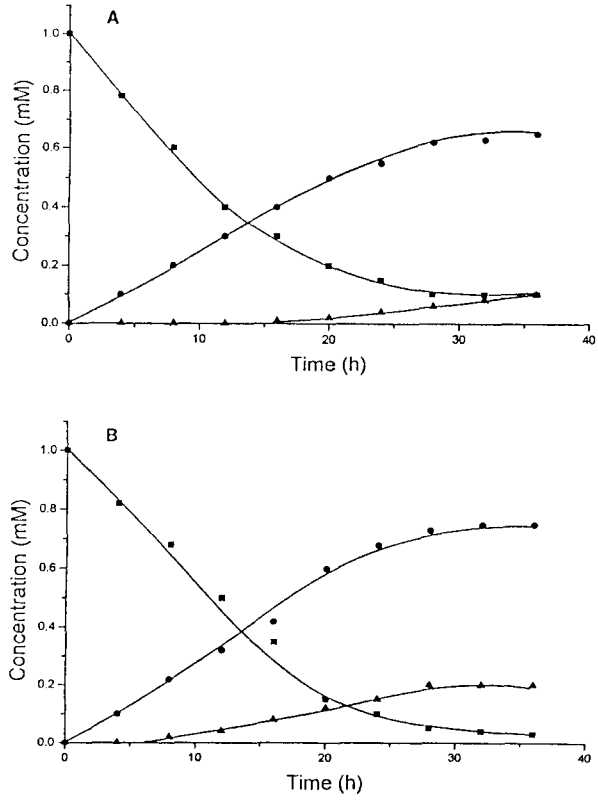
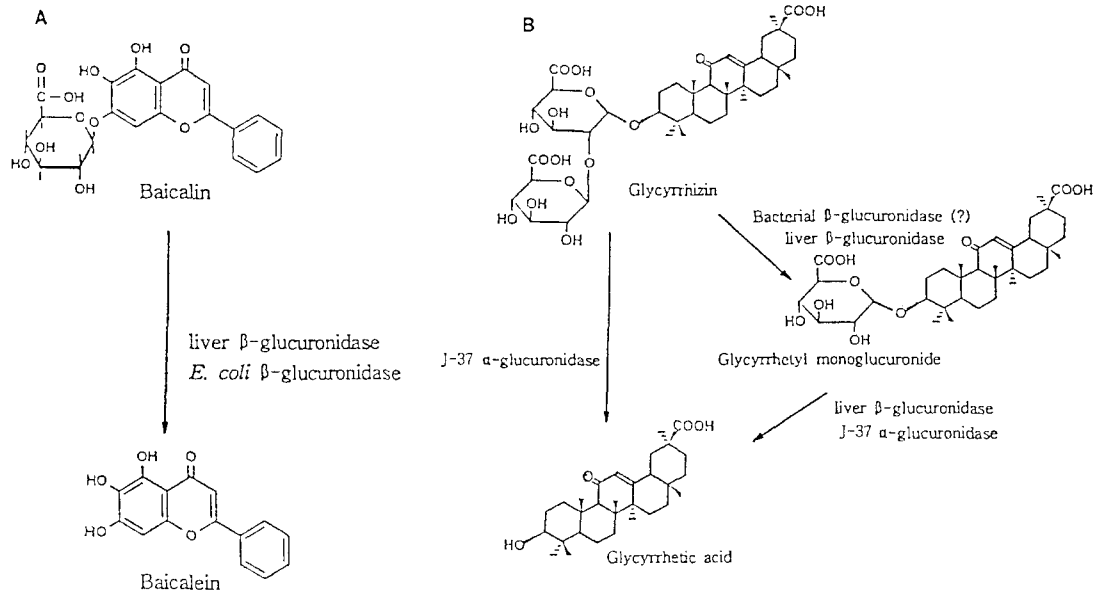


Fig. 2. Metabolic time course of glycyrrhizin by rat liver (A) and bovine (B) β-glucuronidase. The incubated reaction mixture was acidified and extracted with ethylacetate. The upper phase was chromatographed on TLC plate and analyzed by TLC scanner. ■, glycyrrhizin; ●, GAMG; ▲, GA

Table II. Activity of glucuronidases from several sources

	Activity			
	GL → GAMG	GL → GA	GAMG → GA	Baicalin → Baicalein
Rat liver glucuronidase	++++	-	+	++++
Bovine glucuronidase	++	-	+	++++
J-37 glucuronidase	-	++	++++	-
HGU-3 glucuronidase	-	-	-	++++
E.coli glucuronidase	-	-	+	++++

++++, very strongly transformed; +++, strongly transformed; ++, moderately transformed
+, weakly transformed; -, not transformed



Scheme 1. Metabolic pathway baicalin (A) and glycyrrhizin (B) by glucuronidase of human intestinal bacteria and mammalian tissues

were used as the substrate.

When GL was incubated with liver β-glucuronidase of rat and bovine, time course of GL-hydrolysis was measured (Fig. 2). GL was transformed to GA and GAMG by both β-glucuronidases. GL was transformed to GAMG within 10 min. However, little amount of GA was detected within 10 min. It means that GL was transformed to GA via GAMG. Among these substrates, the best substrate of commercial bovine β-glucuronidase and rat liver β-glucuronidase was baicalin, followed by GL and GAMG. GL was more effective than GAMG as the substrate of both enzymes. Thus, by these enzymes, β-glucuronic acid deconjugation (hydrolysis of GL to GAMG) were more selective than that α-glucuronic acid deconjugation (hydrolysis of GAMG to GA).

The best substrate of the β-glucuronidase of commercial *E. coli* (Sigma Chem. Co.) and *E. coli* HGU-3, which was isolated from human intestinal bacteria, were baicalin, followed by GAMG. However, GL was ineffective as the substrate.

The best substrate of *Bacteroides* J-37 glucuronidase was GAMG, followed by GL. GL was transformed to GA by the J-37 glucuronidase but was not to GAMG.

DISCUSSION

Many β-glucuronidases have been reported from bacteria to mammals. Particularly, lysosomal β-glucuronidase transformed GL and baicalien to GAMG/GA and baicalein. *E. coli* β-glucuornidase metabolized baicalin and GAMG to baicalein and GA, respectively, but did not GL. *E. coli* HGU-3 β-glucuronidase hydrolyzed baicalin to baicalein but did not GAMG and GL. The best substrate of all these enymes was p-nitrophenyl-β-D-glucuronide.

However, the substrate specificity of the glucuronidase isolated from J-37 was different to that of the above β-glucuronidase. The best substrate of J-37 glucuronidase was β-glucuronic acid conjugate, GAMG.

When GL was orally or intraveously administered on human as a medicine, metabolites were different in the sera of human subject according to administered routes. By oral administration of GL, GA, not GL, was detected in the sera. By intravenous administration of GL, GAMG, GL and GA were detected in the sera. This GAMG could be mainly produced by lysosomal β-glucuronidase of liver and excreted rapidly into bile.

These results suggest that, as shown in Scheme 1, GL orally administered and GAMG excreted into bile could be metabolized in the human intestine by the α-glucuronidase of human intestinal bacteria, and baicalin could be metabolized to baicalein by β-glucuronidase of human intestinal bacteria.

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