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Metabolism of Saikosaponin c and Naringin by Human Intestinal Bacteria

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By human intestinal bacteria, saikosaponin c was transformed to four metabolites, prosaikogenin E1 (E1) prosaikogenin E2 (E2), prosaikogenin E3 (E3) and saikogenin E. Metabolic time course of saikosaponin c was as follows; in early time, saikosaponin c was converted to E1 and E2, and then these were transformed to saikogenin E via E3. Also, this metabolic pathway was similar to the metabolism of saikosaponin c by rat intestinal bacteria. *Bacteroides* JY-6 and *Bacteroides* YK-4, the bacteria isolated from human intestinal bacteria, could transform saikosaponin c to E via E1 (or E2) and E3. However, these bacteria were not able to directly transform E1 and E2 to saikogenin E. Naringin was mainly transformed to naringenin by human intestinal bacteria. The minor metabolic pathway transformed naringin to naringenin via prunin. By JY-6 or YK-4, naringin was metabolized to naringenin only via prunin.

Key words : *Bacteroides spp.*, Saikosaponin c, Naringin, Intestinal bacteria

INTRODUCTION

In traditional medicine, most of herbal drugs are orally administered as decoctions. Active components of these herbal drugs are inevitably in contact with intestinal microflora in the alimentary tract. Some are transformed by the intestinal bacteria before being absorbed from the gastrointestinal tract. Studies on the metabolsim of these components of these drugs by human intestinal microflora are of a great importance to an understanding of their biological effects (Kim, 1993).

Among herbal medicines, the root of *Bupleurum falcatum* is frequently used in the traditional medication and is one of the most important crude drugs in oriental medicine (Shibata *et al.*, 1970). There have been a lot of reports on its constituents and pharmacological activities since 1960. Shibata *et al.* (1966) and Kubota *et al.* (1967) isolated triterpenoidal saponins from *B. falcatum* which exhibited anti-inflammatory, hepatoprotective, sedative, analgesic and antipyretic effects. After oral administration of saikosaponins of *B. falcatum*, these components should be exposed to gastric juice and mouse intestinal flora in the alimentary tract and some of them could be transformed before absorption into the blood. Particularly, the transformation of saikosaponin c by acid hydrolysis and mouse intestinal bacteria cultured in the Davi's medium was studied by Shimizu *et al.* (1985): saikosaponin c was transformed to saikosaponin h and i by acid hydrolysis and to saikogenin c by mouse intestinal bacteria, respectively. However, we could not obtain any information about the metabolism of saikosaponin c by human intestinal bacteria.

The present paper compared metabolism of saikosaponin c with that of naringin by human intestinal bacteria.

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MATERIALS AND METHODS

Materials

Saikosaponin c, saikogenin E and prosaikogenin E1 (E1), prosaikogenin E2 (E2) and prosaikogenin E3 (E3) were prepared according to our previous method (Kang, 1996). Naringin, naringenin and p-nitrophenyl α-L-rhamnopyranoside (PNR) were purchased from Sigma Chem. Co. (U.S.A.). Prunin (naringin 7-β-D-glucopyranoside) was prepared according to our previous method (Kim *et al.*, 1994). GAM and BL were purchased from Nissui Pharm. Co., ltd. (Japan). The other media were purchased from Difco Co. (U.S.A.).

Thin layer chromatography

TLC for saikosaponin c, E1, E2, E3 and aglycone was performed on silica gel plates (Merck, silica gel 60F-254) as follows; developing solvents A system,

lower phase of $CHCl_3$/methanol/water (65:35:10). TLC for naringin, prunin and naringenin was performed on silica gel plates as follows: developing solvents B system, $CHCl_3$/MeOH (4:1). The quantity of these compounds was assayed with a TLC scanner (Shimadzu CS-920).

Time courses of metabolism of saikosaponin c and naringin by intestinal bacteria

Saikosaponin c metabolizing activities were measured as follows. The assay mixture contained 8 ml of 1 mM saikosaponin c (or naringin) and 0.2 g fresh human fecal bacteria of human or rat (or cultured bacteria) in a final volume of 20 ml of an anaerobic dilution medium. The mixture was incubated at 37°C for 2 days and an aliquot (1 ml) of the reaction mixture was periodically extracted twice with 5 ml of ethylacetate. The ethylacetate fraction was analyzed by TLC.

Isolation of intestinal bacteria metabolizing saikosaponin c

A suspension of the fresh feces of Korean man was diluted 10^5 to 10^8-fold with GAM broth. An aliquot (200 μl) of the 10^7-diluted human feces was inoculated in a BL agar plate, which were anaerobically incubated at 37°C for 4 days. Each colony was incubated in 5 ml of GAM broth containing 0.4% saikosaponin c. The cultured medium was extracted with 5 ml of ethylacetate. After evaporating the ethylacetate fraction, it was analyzed for saikosaponin c, E1, E2 and E3, and saikogenin E by TLC. Two bacteria, JY-6 and YK-4, were isolated. The former has α-rhamnosidase activity but the latter has not it, using PNR as a substrate. Identification of the isolated bacterium was performed according to Bergey's manual (Holdeman *et al.*, 1984). Both JY-6 and YK-4 were *Bacteroides spp.* (Table I).

Table I. Characteristics of *Bacteroides* YK-4 and JY-6 isolated from human intestinal microflora

Characteristic	YK-4	JY-6
Shape	rod	rod
Oxygen	anaerobe	anaerobe
Gram	-	-
MR test	-	-
VP test	+	-
Nitrate reduction	+	+
Indole production	-	-
H_2S production	-	-
Simmons' citrate	-	-
β-Glucosidase	+	+
α-Rhamnosidase	-	+
Urease	-	-
Catalase	+	+

RESULTS AND DISCUSSION

Metabolites of saikosaponin c and naringin by human intestinal bacteria

To investigate the metabolites of saikosaponin c by human intestinal bacteria, saikosaponin c was anaerobically incubated for 18h with a bacterial mixture from human feces. And then the metabolites were extracted with ethylacetate and analyzed by TLC and carbon-13 nuclear magnetic resonance after being separated by preparative TLC.

Four metabolites, two main metabolites (M2 and M 4) and two minor metabolite (M1 and M3) were observed by TLC (Fig. 1). Rf of major metabolites (M2 and M4) were 0.58 and 0.84 on TLC (developing solvent A system), respectively. TLC chromatogram and carbon-13 nuclear magnetic resonance spectra of M4 showed that the chromone nucleus was intact but all glycosyl moieties were missing. Compared with an authentic saikogenin E, this metabolite (M4) was saikogenin E (Nose *et al.*, 1989). Those of M2 showed that the chromone nucleus was intact but one glucosyl moiety was missing. The spectroscopic data agreed with those of prosaikogenin E2 (Nose *et al.*, 1989).

Rf of minor metabolites (M1 and M3) were 0.38 and 0.69 on TLC (developing solvent A system), respectively. We could not obtain sufficiently these metabolites for carbon-13 NMR. TLC chromatogram of M1 was agreed with E1. After hydrolyzing it, TLC chromatograms of aglycone and sugar were saikogenin E and D-glucose, respectively. By these results, M1 seems to be prosaikogenin E1. TLC chromatogram of M3 was coincident with E3. After hydrolyzing M3, TLC chromatograms of aglycone agreed with that of saikogenin E. Sugar of M3 hydrolysate was coincident to D-glucose spot on TLC. By these results, M3 seems to be E3.

Saikosaponin c is the glycoside which has one rhamnosyl group and two glucosyl groups, Naringin is

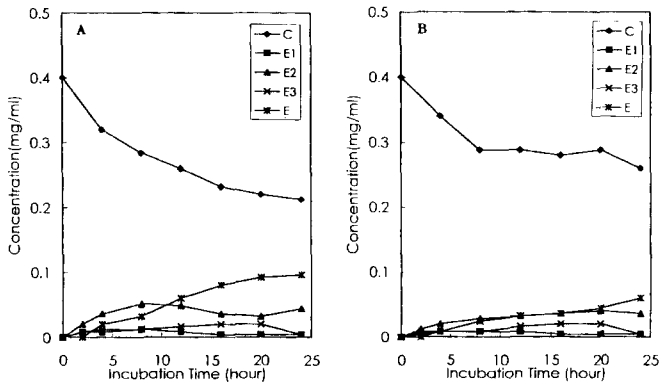


Fig. 1. Time course of metabolism of saikosaponin c by intestinal bacteria of human (A) and rat (B).

also the glycoside one rhamnosyl group and one glucosyl group. Therefore, to compare the metabolic pattern of naringin with that of saikosaponin c by intestinal bacteria, naringin was anaerobically incubated for 9 h with the bacterial mixture of fresh human feces. And then the metabolites were extracted with ethylacetate and analyzed by TLC and carbon-13 nuclear magnetic resonance after being separated by means of silica gel column chromatography. Two metabolites were identified. The major metabolite (>95%) was naringenin and the minor was prunin. These results were coincident with our previous data (Kim *et al.*, 1996).

The time course of metabolism of saikosaponin c and naringin by intestinal bacteria

The time course of transformation of saikosaponin c by human intestinal bacteria was shown in Fig. 2. Saikosaponin c started to be converted to E1 and E2. Thereafter, the major metabolite was E2 and the minor metabolite were E1, E3 and saikogenin E after 6h-incubation with the bacterial mixture. The main metabolite was E2 and saikogenin E after 12h-incubation. Thereafter, main metabolite was saikogenin E after 24h-incubation.

The time course of saikosaponin c metabolism by rat intestinal bacteria was also similar to the metabolic pattern of saikosaponin c by human intestinal bacteria.

The time course of naringin metabolism by human intestinal bacteria was shown in Fig. 3. Naringin, which is a flavonoid having a rhamnosy group and a glucosyl group, started to be converted to naringenin and prunin. The major metabolite (>95%) was naringenin from 3h to 24h. The minor metabolite was prunin, which transformed to naringenin after 24h-incubation with the bacterial mixture.

The metabolic time course of naringin by rat intestinal bacteria was also similar to that of naringin by human intestinal bacteria.

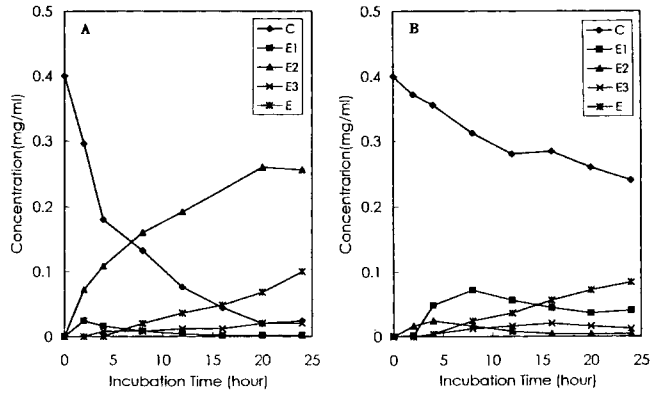


Fig. 3. Time course of metabolism of saikosaponin c by JY-6 (A) and YK-4 (B) isolated from human intestinal bacteria.

The time course of metabolism of saikosaponin c and naringin by the bacteria isolated from human intestinal bacteria, *Bacteroides* JY-6 and *Bacteroides* YK-4

The time course of transformation of saikosaponin c by JY-6 or YK-4 was shown in Fig. 4. By JY-6, saikosaponin c started to be converted to E1 and E2. The major metabolite was E2 and the minor metabolite were E3 and saikogenin E after 8h-incubation with the bacterial mixture. The main metabolite was E2 from 12h to 24h after incubation.

By YK-4, saikosaponin c started to be converted to E1 and E2. However, the major metabolite was E1 and the minor metabolites were E3 and saikogenin E after 12h-incubation with the bacterial mixture. The main metabolite was E1 and saikogenin E after 16h-incubation. The main metabolite was saikogenin after 24h-incubation. The activity of YK-4 transforming E1 to saikogenin E was more potent than that of JY-6. However, using PNR as a substrate, α-rhamnosidase

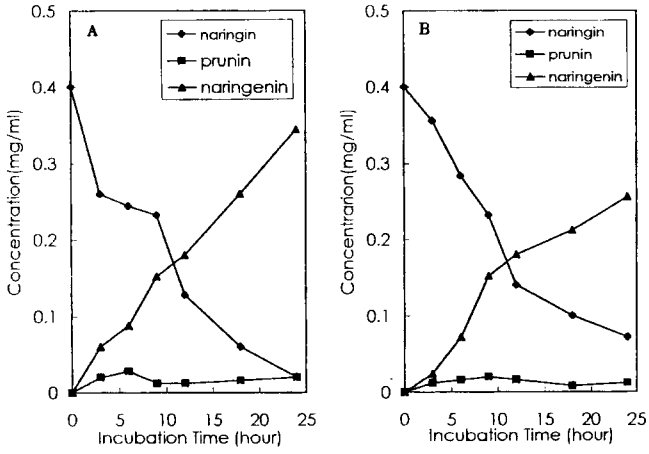


Fig. 2. Time course of metabolism of naringin by intestinal bacteria of human (A) and rat (B).

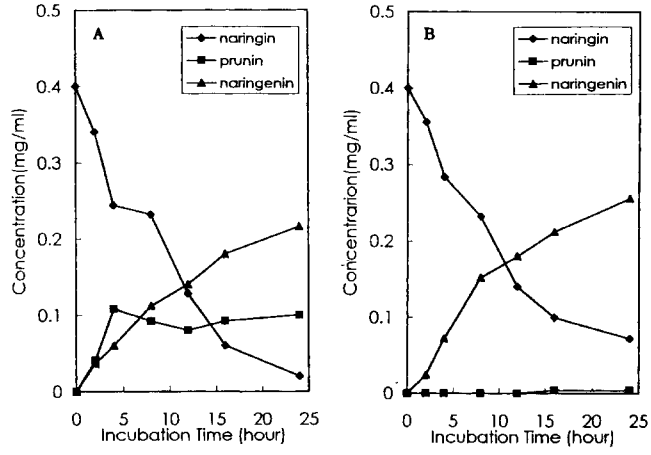
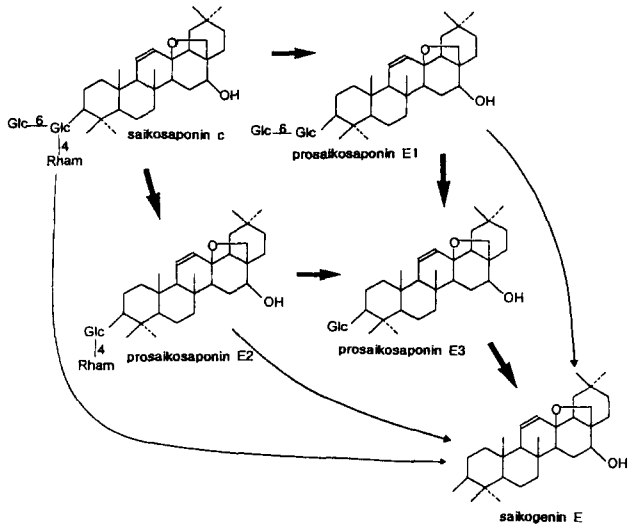


Fig. 4. Time course of metabolism of naringin by JY-6 (A) and YK-4 (B) isolated from human intestinal bacteria.



Scheme 1. Possible metabolic process of saikosaponin c by human intestinal microflora. Main pathway, ➔; minor pathway, →.

activity of JY-6 was higher than that of YK-4. These results suggested that the enzyme hydrolyzing the rhamnosy moiety of saikosaponin c was different to that hydrolyzing PNR on the substrate specificity.

The time course of metabolism of naringin by YK-4 and JY-6 was shown in Fig. 6. By JY-6, naringin started to be converted to naringenin and prunin. The other metabolites were poncirin, ponciretin and pocirenin. The major metabolite was prunin after 3h-incubation. Naringenin transformed from prunin became a main metabolite after 6h-incubation. The other metabolic pathway was reported in the previous paper (Kim *et al.*, 1994).

By YK-4, naringin was converted to naringenin, not prunin. Naringenin only was like to be the metabolite. To clarify the metabolic pathway, we partially purified the β-glucosidase from YK-4 by collection of cells cultured in 1 liter of GAM broth, sonication and ammonium sulfate fractionation (saturated 30~70%), butyl-toyopearl column chromatography (Fr. Nos. 40~45). The partially purified enzyme did not transformed naringin to naringenin. The fractions of butyl-toyopearl column chromatography which did not contain β-glucosidase (Fr. Nos. 47-52) could metabolize naringin to prunin. However, these fractions have little the activity hydrolyzing PNR. By mixing these fractions with β-glucosidase-active fractions, naringin could be transformed to naringenin. These results also suggested that YK-4 α-rhamnosidase is different from that of JY-6 on substrate specificity.

Metabolic pathway of saikosaponin c and naringin

When saikosaponin c was incubated with human intestinal bacteria, four metabolites (E1, E2, E3 and saikogenin E) were produced. The metabolic pathway of saikosaponin c by human intestinal bacteria was proposed as follows. In early time, saikosaponin c was converted to E1 and E2, and then to saikogenin E via E3 (Scheme 1). The activity of each metabolic process was shown in Table II. Thus, saikosaponin c was converted to E1 by hydrolyzing its rhamnosyl moiety and then to E3 by hydrolyzing glucosyl moiety of E1. Thereafter, E3 was converted to saikogenin E by hydrolyzing glucosyl moiety of E3. In addition, saikosaponin c was converted E2 by hydrolyzing its glucosyl moiety and then E2 was weakly to E3 by hydrolyzing rhamnosyl moiety of E2. E3 was transformed to saikogenin E by hydrolyzing glucosyl moiety of E3. The metabolic pathway of saikosaponin c by rat intestinal bacteria was also similar to the above pathway by human intestinal bacteria. Another metabolic pathway was the process that directly transformed E1 and E2 to E. This metabolic pathway seems to be proceeded very weakly. However, saikosaponin c seems to be not transformed to E.

JY-6 and YK-4, the bacteria isolated from human intestinal, could transform saikosaponin c to E via E1 (or E2) and E3. However, these bacteria could not transform directly E1 and E2 to E. These bacteria did not produce endo-β-glucosidase. JY-6 could potently transform saikosaponin c to E2 but YK-4 could metabolize potently saikosaponin to E1. These results also suggested that YK-4 α-rhamnosidase is different from that of JY-6 on substrate specificity like the case of the metabolism of saikosaponin c. The other reaction activity was similar between YK-4 and JY-6.

Naringin was mainly transformed to naringenin by human intestinal bacteria. The minor metabolic path-

Table II. Glycosidation activity of intestinal bacteria on the metabolism of saikosaponin c and naringin

Source	Activity[1)]									
	C→E1	C→E2	C→E	E1→E3	E2→E3	E1→E	E2→E	naringin→ prunin	prunin→ naringenin	naringin→ naringenin
Human intestinal bacteria	++	+++	±	+++	++	+	+	+++	+++	+
Rat intestinal bacteria	++	+++	±	+++	++	+	+	+++	+++	+
Bacteroides JY-6	+	+++	-	+++	+	-	-	+++	+	-
Bacteroides YK-4	++	+	-	+++	+++	-	-	+	+++	-

1:+++, strongly transformed;++, moderately transformed;+, weakly transformed;±, nearly not transformed;-, not transformed.

way was to transform naringin to naringenin via prunin.

By JY-6, naringin was metabolized only to naringenin via prunin. However, YK-4 was likely to directly transform naringin to naringenin (not via prunin). We partially purified β-glucosidase from YK-4. The enzyme could not transform naringin to naringenin. α-Rhamnosidase partially purified from YK-4 transformed naringin to prunin. Therefore, β-glucosidase mixed with the α-rhamnosidase transformed naringin to naringenin via prunin. Thus, we thought that YK-4 is likely to transform naringin to naringenin, because YK-4 α-rhamnosidase activity was too weak and its β-glucosidase was potent. However, YK-4 could not transform directly naringin to naringenin. The α-rhamnosidase of YK-4 nearly did not has the activity hydrolyzing PNR. However, the α-rhamnosidase of JY-6 has the activity potently hydrolyzing PNR. α-Rhamnosidase of YK-4 was different from that of JY-6 on the substrate specificity.

By intestinal bacteria, saikosaponin c and naringin were easily transformed to their aglycones. We insist that these metabolic processes are important in the pharmacological effect of traditional medicines.

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