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Intestinal Bacterial Hydrolysis is Required for the Appearance of Compound K in Rat Plasma after Oral Administration of Ginsenoside Rb_1 from *Panax ginseng*

TERUAKI AKAO, HIROAKI KIDA*, MATAO KANAOKA, MASAO HATTORI* AND KYOICHI KOBASHI

*Faculty of Pharmaceutical Sciences and *Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan*

Abstract

Ginsenoside Rb_1 from *Panax ginseng* root is transformed into compound K via ginsenosides Rd and F_2 by intestinal bacterial flora. Among 31 defined intestinal strains from man, only Eubacterium sp. A-44 transformed ginsenoside Rb_1 into compound K via ginsenoside Rd. The ginsenoside Rb_1-hydrolysing enzyme isolated from Eubacterium sp. A-44 was identical to a previously purified geniposide-hydrolysing β-D-glucosidase.

When ginsenoside Rb_1 (200 mg kg^{-1}) was administered orally to germ-free rats, neither compound K nor any other metabolite was detected in the plasma, intestinal tract or cumulative faeces 7 or 15 h after administration. Most of the ginsenoside Rb_1 administered was recovered from the intestinal tract, especially the caeca, and cumulative faeces indicating poor absorption of ginsenoside Rb_1. When ginsenoside Rb_1 was administered orally to gnotobiote rats mono-associated with Eubacterium sp. A-44, a significant amount of compound K was detected in the plasma and considerable amounts were found in the caecal contents and cumulative faeces 7 and 15 h after administration. A small amount of ginsenoside Rb_1 was detected in the caecal contents only 7 h after administration.

These results indicate that orally administered ginsenoside Rb_1 is poorly absorbed from the gut but that its metabolite compound K, produced by ginsenoside Rb_1-hydrolysing bacteria such as Eubacterium sp. A-44 in the lower part of intestine, is absorbed.

Ginseng, the root of *Panax ginseng*, is an important drug used in Oriental medicines. Its main constituents are glycosides of dammarene-type triterpenes, protopanaxadiol and protopanaxatriol (Shibata et al 1985). Pharmacological and biological activity has been reported for various ginsenosides (Lacaille-Dubois & Wagner 1966; Gillis 1997). Poor absorption of ginsenosides Rg_1 and Rb_2 has been observed (Odani et al 1983a; Takino 1994) and after oral administration to rats ginsenoside Rb_1 (protopanaxadiol type), the most abundant constituent of ginsenosides, could not be detected in the serum by thin-layer chromatography (TLC) and only an extremely low proportion (0·05%) of the dose was excreted in the urine within 24 h (Odani et al 1983b). In man ginsenoside Rb_1 was undetectable in the plasma by high-performance liquid chromatography (HPLC) or by a sensitive enzyme immunoassay (EIA), developed by Kanaoka et al (1992), for ginsenoside Rb_1 after ingestion of red ginseng powder (Kato et al 1990).

Ginsenosides are transformed into deglucosylated metabolites by intestinal bacteria in-vitro and in-vivo (Odani et al 1983c; Karikura et al 1991; Kanaoka et al 1994; Takino 1994; Hasegawa et al 1996) and ginsenoside Rb_1 is hydrolysed to compound K, its main metabolite, via ginsenosides Rd and F_2 by intestinal flora in rat and man (Figure 1) (Karikura et al 1991; Kanaoka et al 1994). Compound K has been detected in the plasma after oral administration of ginsenoside Rb_1 (Akao et al 1998) or ginseng saponins (Hasegawa et al 1996) to rats, albeit some time later, whereas rapid absorption occurs after oral administration of compound K itself (Akao et al 1998). These results suggest that unabsorbed ginsenoside Rb_1 is transformed into compound K by intestinal bacteria in the lower part of the rat intestine and that compound K is then absorbed.

Correspondence: T. Akao, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan.

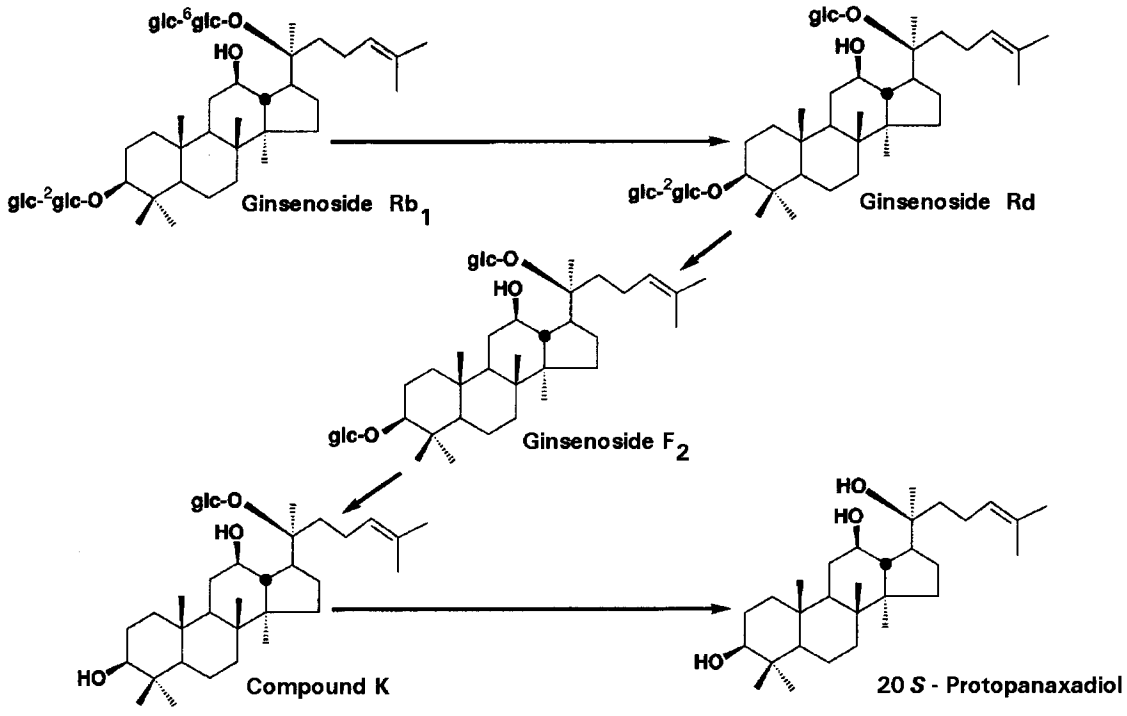

Figure 1. Metabolism of ginsenoside Rb_1 by intestinal bacterial flora from rat and man.

In this study we have screened 31 defined strains of intestinal bacteria from man for ginsenoside Rb_1-metabolizing activity and found only one strain, Eubacterium sp. A-44, capable of transforming ginsenoside Rb_1 into compound K. After preparing gnotobiote rats mono-associated with Eubacterium sp. A-44, we clarified in an in-vivo experiment that this ginsenoside Rb_1-hydrolysing bacterium was necessary for the appearance of compound K in rat plasma after oral administration of ginsenoside Rb_1.

Materials and Methods

Chemicals

Reagents for the compound K enzyme immunoassay (EIA) were prepared or purchased as described previously (Akao et al 1998). Ginsenosides Rb_1 and Rd were kindly supplied by Nikkan Korai Ginseng (Kobe, Japan). Compound K was prepared by treating ginsenoside Rb_1 with crude naringinase (Akao et al 1998). General anaerobic medium (GAM) was purchased from Nissui Seiyaku (Tokyo, Japan).

Bacterial strains and culture

Bifidobacterium sp. strain SEN (Akao et al 1994a), *Clostridium innocuum* ES24-06 (Hattori et al 1985), Eubacterium sp. A-44 (Kobashi et al 1986), Eubacterium sp. strain BAR (Che et al 1991) and Ruminococcus sp. PO1-3 (Hattori et al 1985) have previously been isolated from the faeces of man. Other defined strains of intestinal bacteria from man were provided by Professor Emeritus T. Mitsuoka, The University of Tokyo. Each strain was cultured in semi-solid GAM agar and maintained at 4°C.

Thin-layer chromatography (TLC)

Silica gel type 60 plates (Merck, Darmstadt, Germany) were used for determination of ginsenosides Rb_1 and Rd (mobile phase $CHCl_3$–methanol–H_2O, 65:35:10, lower layer), and of compound K (mobile phase $CHCl_3$–methanol, 4:1) (Kanaoka et al 1994). Spots were detected by spraying with phosphomolybdic acid reagent and heating; densitometric scanning (Shimadzu CS-9000) was performed at 600 nm.

Screening of defined bacterial strains for metabolism of ginsenoside Rb_1

Each precultured bacterium (10 μL) was added to GAM broth (10 mL) and cultured in an anaerobic incubator at 37°C for 24 h. The individual precipitates obtained by centrifugation were washed with saline and suspended with 1·5 mL 50 mM potassium phosphate buffer (pH 7·2). Each bacterial suspension (100 μL) was incubated with 1 mM ginsenoside Rb_1 at 37°C for 24 h under anaerobic conditions. The reaction mixture was extracted with butanol (100 μL) and metabolites such as

ginsenoside Rd and compound K were analysed by TLC as described above.

Enzyme assay

β-D-Glucosidase activity was measured as described previously (Yan et al 1995) using *p*-nitrophenyl β-D-glucopyranoside as substrate. Arylsulphotransferase was assayed using *p*-nitrophenyl sulphate and tyramine as the donor and acceptor, respectively, by a minor modification of the method of Kim et al (1986). To measure ginsenoside Rb_1-hydrolysing activity reaction mixture containing ginsenoside Rb_1 (0·5 μmol) and enzyme solution in potassium phosphate buffer (50 mM, pH 6·3; 200 μL) was incubated at 37°C for 20–60 min then the reaction was stopped by adding 200 μL water-saturated butanol. The amounts of hydrolysates were determined by TLC as described above. Protein was determined by the method of Lowry et al (1951) using bovine serum albumin (BSA) as a standard.

Partial purification of a ginsenoside Rb_1-hydrolysing β-D-glucosidase from Eubacterium sp. A-44

The method of purification of the enzyme from the extract of Eubacterium sp. A-44, by column chromatography on Butyl-Toyopearl 650M, Sephacryl S-300 and hydroxyapatite, was fundamentally the same as for geniposide-hydrolysing β-D-glucosidase (Yan et al 1995).

Animals, treatment and sampling

Male Wistar germ-free rats (WA/Jic, 5–6 weeks; CLEA Japan, Tokyo, Japan) were individually maintained in metabolic cages under germ-free conditions and fasted overnight before the experiments. Autoclaved water and sterilized standard laboratory chow (CE-2, X-ray-treated, CLEA Japan) were otherwise freely available. Six germ-free rats were infected with Eubacterium sp. A-44 (2 mL medium cultured overnight) on the first and third days to produce the gnotobiote rats. One week later sterile ginsenoside Rb_1 dissolved in pure water was administered orally to six germ-free rats and to the gnotobiote rats at a dose of 200 mg kg^{-1}. The cumulative faeces were collected 7 h (three rats) and 15 h (three rats) after administration. Blood samples from the abdominal vein and the gastrointestinal tract were taken under pentobarbital anaesthesia 7 h (three rats) and 15 h (three rats) after administration. Plasma samples were prepared by centrifugation of the heparinized blood and stored at −20°C until use.

Determination of compound K in plasma

Plasma (50 μL) was extracted with methanol (2 × 200 μL) and the extract was dried in-vacuo and redissolved in methanol (20 μL). Buffer A (20 mM phosphate-buffered saline, pH 6·8, 80 μL, containing 0·1% BSA, 0·1% NaN_3 and 0·001% $MgCl_2$) was added and the concentration of compound K was measured by an EIA method described elsewhere (Akao et al 1998).

Determination of ginsenoside Rb_1 and compound K in intestinal contents and faeces

Suspensions of the contents of the small intestine, caecum and colon–rectum, and faecal suspensions were extracted with an equal volume of water-saturated butanol. A sample of the butanol layer was analysed by TLC as described above to determine the ginsenoside Rb_1 and compound K contents.

Results and Discussion

Screening of defined strains of intestinal bacteria from man for metabolism of ginsenoside Rb_1

Of 31 strains of intestinal bacteria only one, Eubacterium sp. A-44, metabolized ginsenoside Rb_1 (Table 1). This bacterium transformed ginsenoside Rb_1 into compound K and ginsenoside Rd, in a manner similar to that of faecal flora from rat and man (Karikura et al 1991; Kanaoka et al 1994). Although Eubacterium is one of the most predominant genera of the intestinal flora of man, few strains seem to be able to hydrolyse ginsenoside Rb_1, because a 3% probability was found on screening for this ability. This is supported by the finding that only one species, *Prevotella oris*, isolated from human faeces by Hasegawa et al (1997) was a ginsenoside Rb_1-metabolizing bacterium. Ginsenoside Rb_1-hydrolysing activity (9·6 nmol min^{-1} mg^{-1}) has also been observed in a crude extract of Eubacterium sp. A-44 which contained three different kinds of β-D-glucosidase (Akao et al 1994b) with geniposide-hydrolysing (Yan et al 1995) or other activity. These enzymes were clearly separated by column chromatography on Butyl-Toyopearl 650M. The first eluted peak fraction hydrolysed *p*-nitrophenyl β-D-glucopyranoside and geniposide. This fraction also hydrolysed ginsenoside Rb_1 to ginsenoside Rd and compound K, but with less activity (one third of that of *p*-nitrophenyl β-D-glucopyranoside and one half that of geniposide), but did not hydrolyse ginsenoside Rg_1 (protopanaxatriol type). This ginsenoside Rb_1- and geniposide-hydrolysing activity was further co-purified by column chromatography on Sephacryl S-300 and hydroxyapatite. These results indicate

Table 1. Intestinal bacterial strains from man used to test the metabolism of ginsenoside Rb_1 (GRb_1) with percentages of the recovered substrate.

Intestinal bacteria	GRb_1†
Bacteroides fragilis ss thetaotus	87·5
B. fragilis ss vulgatus	91·6
Bifidobacterium adolescentis	94·3
B. angulatum	82·7
B. bifidum a E319	93·9
B. breve S-2 kz 1287	83·3
B. longum IV-55	85·9
B. pseudolongum PNC-2-9-G	88·3
B. sp. SEN	87·1
Clostridium butyricum	85·4
C. innocuum ES 24-06	89·6
C. innocuum KZ-633	84·2
C. perfringens TO-23	87·2
Enterococcus faecalis II-136	89·4
Eubacterium aerofaciens	84·5
Eubacterium sp. A-44	N.D.*
E. sp. BAR	89·1
Fusobacterium nucleatum	91·4
Klebsiella pneumoniae ATCC 13883	83·4
Lactobacillus acidophilus ATCC 4356	91·8
L. brevis II-46	91·0
L. fermentum ATCC 9338	83·5
L. plantarum ATCC 14927	95·4
L. xylosus ATCC 15775	84·0
Peptostreptococcus anaerobius 0240	82·4
P. intermedius EBF77/25	81·6
Proteus mirabilis S2	86·9
Ruminococcus sp. PO1-3	93·0
Veilloneella parvula ss parvula ATCC 10790	91·7

†No metabolites were detected for all of the intestinal bacteria except E.sp.A-44. *Metabolites ginsenoside Rd (16·8%) and compound K (70·3%) were detected.

that the previously purified enzyme capable of hydrolysing geniposide (Yan et al 1995) has broad substrate specificity and also hydrolyses ginsenoside Rb_1 to compound K via ginsenoside Rd. We have recently clarified that this enzyme hydrolyses saikosaponins a, b_1, b_2 and d, which are resistant to various other β-D-glucosidases, to the corresponding prosaikogenins (Kida et al 1997).

Eubacterium sp. A-44 is required for the appearance of compound K in plasma after oral administration of ginsenoside Rb_1

Although the caecal contents of germ-free rats showed no β-D-glucosidase or arylsulphate sulphotransferase activity (Table 2), this activity was present to a significant extent in the caecal contents of gnotobiote rats infected with Eubacterium sp. A-44 which produces two unique enzymes, arylsulphotransferase (Kim et al 1986) and ginsenoside Rb_1-hydrolysing β-D-glucosidase. Thus, Eubacterium sp. A-44 inhabited the intestinal tracts of the gnotobiote rats produced during this study. After oral administration of 200 mg kg^{-1} ginsenoside Rb_1 to the germ-free rats, none of its intestinal bacterial metabolites, including compound K, were detectable in their intestinal tracts or cumulative faeces. Ginsenoside Rb_1 itself was detected mostly in the caeca. More than 90% of the ginsenoside Rb_1 administered was recovered from the intestinal tracts and cumulative faeces of the rats tested 7 h after administration, and more than 70% from those of the rats tested after 15 h (Table 3). These results indicate that orally administered ginsenoside Rb_1 is excreted in the faeces without being metabolized and that it is not absorbed from the intestinal tracts of the germ-free rats. They also confirm earlier suggestions of poor absorption of ginsenoside Rb_1 on the basis of previous reports (Odani et al 1983b; Kato et al 1990; Hasegawa et al 1996, 1997) that ginsenoside Rb_1 was undetectable in plasma from mice, rats and man after oral administration. After oral administration to the gnotobiote rats significant amounts of ginsenoside Rb_1 and compound K were detected in the rats' intestinal tracts and cumulative faeces after 7 h, and a considerable amount of compound K, but no ginsenoside Rb_1, was detected in their caeca and faeces after 15 h (Table 3). Only a small amount of ginsenoside Rd, one of the other intestinal bacterial metabolites, was detected. Thus, ginsenoside Rb_1 had mostly been transformed into compound K in the caeca and colons of the gnotobiote rats within 7 h of administration, which is compatible with the finding that ginsenoside Rb_1 almost disappeared from the digestive tracts of conventional rats within 6 h of oral administration at a dose of 100 mg kg^{-1} (Odani et al 1983b). However, it seems that not all the compound K produced is absorbed from the gut, because excretion of an appreciable amount into the faeces occurred 15 h after administration. This suggests that compound K is slowly absorbed from the intestinal tract.

Although compound K has appeared at concentrations of 10–100 ng mL^{-1} in the plasma of

Table 2. β-D-Glucosidase and arylsulphotransferase activity of the caecal contents of germ-free and gnotobiote rats.

Animal	Time (h)	Enzyme activity (nmol min^{-1} mg^{-1})	
		β-D-Glucosidase	Arylsulphotransferase
Germ-free rats	7	N.D.	N.D.
	15	N.D.	N.D.
Gnotobiote rats	7	5·57 ± 3·37	4·40 ± 1·97
	15	6·63 ± 3·97	4·80 ± 2·09

Values are means ± s.d. (n = 3) 7 and 15 h after oral administration of ginsenoside Rb_1. N.D., not detected.

Table 3. Recovery of ginsenoside Rb_1, and the compound K content of the intestinal tracts and cumulative faeces of germ-free and gnotobiote rats 7 and 15 h after oral administration of ginsenoside Rb_1.

		Germ-free rats		Gnotobiote rats	
		7 h	15 h	7 h	15 h
Small intestine	Ginsenoside Rb_1	13·5	2·9	N.D.	N.D.
	Compound K	N.D.	N.D.	5·6	6·7
Caecum	Ginsenoside Rb_1	67·3	51·7	32·0	N.D.
	Compound K	N.D.	N.D.	9·3	19·2
Colon + rectum	Ginsenoside Rb_1	4·5	7·4	3·5	N.D.
	Compound K	N.D.	N.D.	0·6	2·1
Faeces	Ginsenoside Rb_1	5·6	8·8	2·5	N.D.
	Compound K	N.D.	N.D.	0·7	18·9

Data are mean recoveries (%) of the dose administered (n = 3). N.D., not detected.

conventional rats within 4–24 h of oral administration of 200 mg kg^{-1} ginsenoside Rb_1 (Akao et al 1998), no compound K was detected in the plasma of the germ-free rats either 7 or 15 h after oral administration of the same dose of ginsenoside Rb_1. Compound K was detected at concentrations of 4·8 and 83·4 ng mL^{-1} in the plasma of the gnotobiote rats 7 and 15 h, respectively, after oral administration of ginsenoside Rb_1. The concentration of compound K in the plasma of gnotobiote rats was similar to that found in conventional rats. These findings accord with the above results showing that compound K was produced in the intestinal tracts of the gnotobiote rats after oral administration of ginsenoside Rb_1, but that no compound K was generated by the germ-free rats.

In conclusion, ginsenoside Rb_1-hydrolysing bacteria such as Eubacterium sp. A-44 are essential for the appearance of compound K in the plasma after oral administration of ginsenoside Rb_1 to rats, and this probably applies to man also. Orally administered ginsenoside Rb_1 reaches the lower part of the intestinal tract without being absorbed, is transformed into compound K and other metabolites by intestinal anaerobes, and these metabolites are then absorbed. *Panax ginseng* root (ginseng) contains ginsenosides Rb_2, Rb_3, Rc and Rd, and ginsenoside Rb_1. These are all glycosides of protopanaxadiol, and these saponins are also transformed mainly to compound K by intestinal bacterial flora (Karikura et al 1991; Kanaoka et al 1994; Hasegawa et al 1996). Accordingly, when man ingests ginseng, compound K seems to be one of the main compounds absorbed.

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