

# Intestinal Bacterial Hydrolysis is Required for the Appearance of Compound K in Rat Plasma after Oral Administration of Ginsenoside Rb<sub>1</sub> from *Panax ginseng*

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## Abstract

Ginsenoside Rb<sub>1</sub> from *Panax ginseng* root is transformed into compound K via ginsenosides Rd and F<sub>2</sub> by intestinal bacterial flora. Among 31 defined intestinal strains from man, only *Eubacterium* sp. A-44 transformed ginsenoside Rb<sub>1</sub> into compound K via ginsenoside Rd. The ginsenoside Rb<sub>1</sub>-hydrolysing enzyme isolated from *Eubacterium* sp. A-44 was identical to a previously purified geniposide-hydrolysing  $\beta$ -D-glucosidase.

When ginsenoside Rb<sub>1</sub> (200 mg kg<sup>-1</sup>) 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<sub>1</sub> administered was recovered from the intestinal tract, especially the caeca, and cumulative faeces indicating poor absorption of ginsenoside Rb<sub>1</sub>. When ginsenoside Rb<sub>1</sub> was administered orally to gnotobiotic 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<sub>1</sub> was detected in the caecal contents only 7 h after administration.

These results indicate that orally administered ginsenoside Rb<sub>1</sub> is poorly absorbed from the gut but that its metabolite compound K, produced by ginsenoside Rb<sub>1</sub>-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<sub>1</sub> and Rb<sub>2</sub> has been observed (Odani et al 1983a; Takino 1994) and after oral administration to rats ginsenoside Rb<sub>1</sub> (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<sub>1</sub> 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<sub>1</sub> after ingestion of red ginseng powder (Kato et al 1990).

Ginsenosides are transformed into deglycosylated 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<sub>1</sub> is hydrolysed to compound K, its main metabolite, via ginsenosides Rd and F<sub>2</sub> 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<sub>1</sub> (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<sub>1</sub> is transformed into compound K by intestinal bacteria in the lower part of the rat intestine and that compound K is then absorbed.

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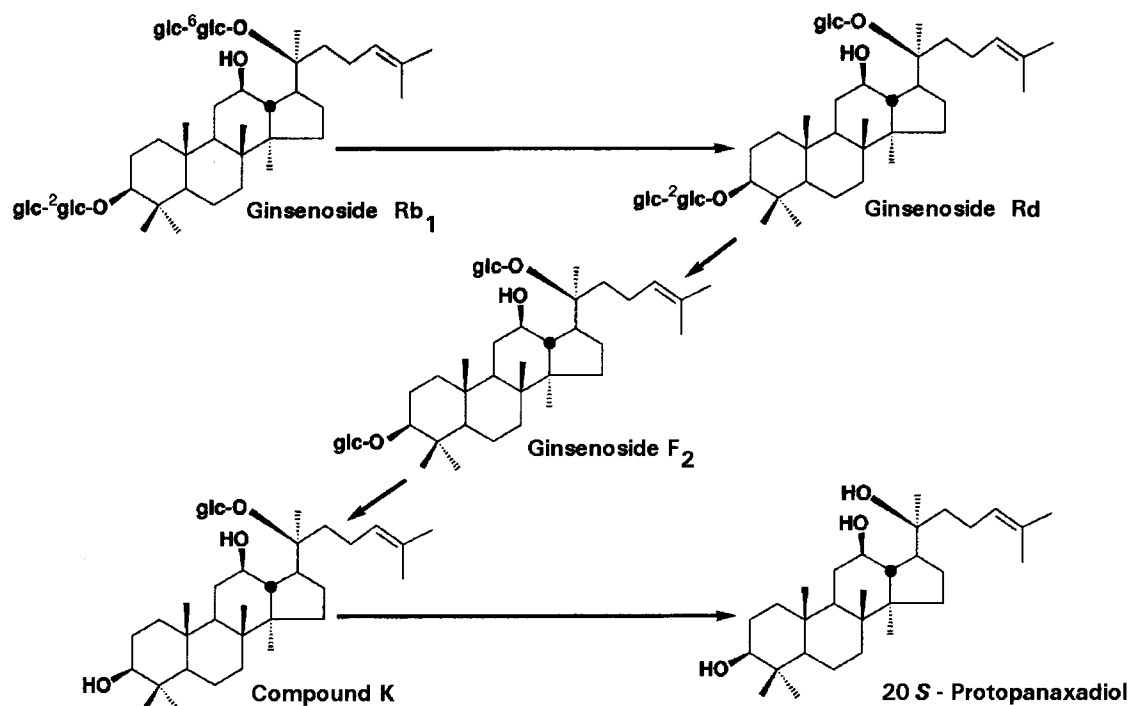


Figure 1. Metabolism of ginsenoside Rb<sub>1</sub> by intestinal bacterial flora from rat and man.

In this study we have screened 31 defined strains of intestinal bacteria from man for ginsenoside Rb<sub>1</sub>-metabolizing activity and found only one strain, *Eubacterium* sp. A-44, capable of transforming ginsenoside Rb<sub>1</sub> into compound K. After preparing gnotobiotic rats mono-associated with *Eubacterium* sp. A-44, we clarified in an in-vivo experiment that this ginsenoside Rb<sub>1</sub>-hydrolysing bacterium was necessary for the appearance of compound K in rat plasma after oral administration of ginsenoside Rb<sub>1</sub>.

## 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<sub>1</sub> and Rd were kindly supplied by Nikkan Korai Ginseng (Kobe, Japan). Compound K was prepared by treating ginsenoside Rb<sub>1</sub> 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<sub>1</sub> and Rd (mobile phase CHCl<sub>3</sub>-methanol-H<sub>2</sub>O, 65:35:10, lower layer), and of compound K (mobile phase CHCl<sub>3</sub>-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<sub>1</sub>

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<sub>1</sub> 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

$\beta$ -D-Glucosidase activity was measured as described previously (Yan et al 1995) using *p*-nitrophenyl  $\beta$ -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<sub>1</sub>-hydrolysing activity reaction mixture containing ginsenoside Rb<sub>1</sub> (0.5  $\mu$ mol) and enzyme solution in potassium phosphate buffer (50 mM, pH 6.3; 200  $\mu$ L) was incubated at 37°C for 20–60 min then the reaction was stopped by adding 200  $\mu$ L 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<sub>1</sub>-hydrolysing $\beta$ -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  $\beta$ -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 gnotobiotic rats. One week later sterile ginsenoside Rb<sub>1</sub> dissolved in pure water was administered orally to six germ-free rats and to the gnotobiotic rats at a dose of 200 mg kg<sup>-1</sup>. 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  $\mu$ L) was extracted with methanol (2  $\times$  200  $\mu$ L) and the extract was dried in-vacuo and redissolved in methanol (20  $\mu$ L). Buffer A (20 mM phosphate-buffered saline, pH 6.8, 80  $\mu$ L, containing 0.1% BSA, 0.1% NaN<sub>3</sub> and 0.001% MgCl<sub>2</sub>) was added and the concentration of compound K was measured by an EIA method described elsewhere (Akao et al 1998).

#### Determination of ginsenoside Rb<sub>1</sub> 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<sub>1</sub> and compound K contents.

## Results and Discussion

#### Screening of defined strains of intestinal bacteria from man for metabolism of ginsenoside Rb<sub>1</sub>

Of 31 strains of intestinal bacteria only one, *Eubacterium* sp. A-44, metabolized ginsenoside Rb<sub>1</sub> (Table 1). This bacterium transformed ginsenoside Rb<sub>1</sub> 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<sub>1</sub>, 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<sub>1</sub>-metabolizing bacterium. Ginsenoside Rb<sub>1</sub>-hydrolysing activity (9.6 nmol min<sup>-1</sup> mg<sup>-1</sup>) has also been observed in a crude extract of *Eubacterium* sp. A-44 which contained three different kinds of  $\beta$ -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  $\beta$ -D-glucopyranoside and geniposide. This fraction also hydrolysed ginsenoside Rb<sub>1</sub> to ginsenoside Rd and compound K, but with less activity (one third of that of *p*-nitrophenyl  $\beta$ -D-glucopyranoside and one half that of geniposide), but did not hydrolyse ginsenoside Rg<sub>1</sub> (protopanaxatriol type). This ginsenoside Rb<sub>1</sub>- and geniposide-hydrolysing activity was further copurified 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<sub>1</sub> (GRb<sub>1</sub>) with percentages of the recovered substrate.

Intestinal bacteria	GRb <sub>1</sub> †
<i>Bacteroides fragilis</i> ss thetaotus	87.5
<i>B. fragilis</i> ss vulgatus	91.6
<i>Bifidobacterium adolescentis</i>	94.3
<i>B. angulatum</i>	82.7
<i>B. bifidum</i> a E319	93.9
<i>B. breve</i> S-2 kz 1287	83.3
<i>B. longum</i> IV-55	85.9
<i>B. pseudolongum</i> PNC-2-9-G	88.3
<i>B. sp.</i> SEN	87.1
<i>Clostridium butyricum</i>	85.4
<i>C. innocuum</i> ES 24-06	89.6
<i>C. innocuum</i> KZ-633	84.2
<i>C. perfringens</i> TO-23	87.2
<i>Enterococcus faecalis</i> II-136	89.4
<i>Eubacterium aerofaciens</i>	84.5
<i>Eubacterium sp.</i> A-44	N.D.*
<i>E. sp.</i> BAR	89.1
<i>Fusobacterium nucleatum</i>	91.4
<i>Klebsiella pneumoniae</i> ATCC 13883	83.4
<i>Lactobacillus acidophilus</i> ATCC 4356	91.8
<i>L. brevis</i> II-46	91.0
<i>L. fermentum</i> ATCC 9338	83.5
<i>L. plantarum</i> ATCC 14927	95.4
<i>L. xylosum</i> ATCC 15775	84.0
<i>Peptostreptococcus anaerobius</i> 0240	82.4
<i>P. intermedius</i> EBF77/25	81.6
<i>Proteus mirabilis</i> S2	86.9
<i>Ruminococcus sp.</i> PO1-3	93.0
<i>Veillonella parvula</i> 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<sub>1</sub> to compound K via ginsenoside Rd. We have recently clarified that this enzyme hydrolyses saikosaponins a, b<sub>1</sub>, b<sub>2</sub> and d, which are resistant to various other  $\beta$ -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<sub>1</sub>*

Although the caecal contents of germ-free rats showed no  $\beta$ -D-glucosidase or arylsulphate sulphotransferase activity (Table 2), this activity was present to a significant extent in the caecal contents of gnotobiotic rats infected with *Eubacterium sp.* A-44 which produces two unique enzymes, arylsulphotransferase (Kim et al 1986) and ginsenoside Rb<sub>1</sub>-hydrolysing  $\beta$ -D-glucosidase. Thus, *Eubacterium sp.* A-44 inhabited the intestinal tracts of the gnotobiotic rats produced during this study. After oral administration of 200 mg kg<sup>-1</sup> ginsenoside Rb<sub>1</sub>

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<sub>1</sub> itself was detected mostly in the caeca. More than 90% of the ginsenoside Rb<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> on the basis of previous reports (Odani et al 1983b; Kato et al 1990; Hasegawa et al 1996, 1997) that ginsenoside Rb<sub>1</sub> was undetectable in plasma from mice, rats and man after oral administration. After oral administration to the gnotobiotic rats significant amounts of ginsenoside Rb<sub>1</sub> 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<sub>1</sub>, 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<sub>1</sub> had mostly been transformed into compound K in the caeca and colons of the gnotobiotic rats within 7 h of administration, which is compatible with the finding that ginsenoside Rb<sub>1</sub> almost disappeared from the digestive tracts of conventional rats within 6 h of oral administration at a dose of 100 mg kg<sup>-1</sup> (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<sup>-1</sup> in the plasma of

Table 2.  $\beta$ -D-Glucosidase and arylsulphotransferase activity of the caecal contents of germ-free and gnotobiotic rats.

Animal	Time (h)	Enzyme activity (nmol min <sup>-1</sup> mg <sup>-1</sup> )	
		$\beta$ -D-Glucosidase	Arylsulphotransferase
Germ-free rats	7	N.D.	N.D.
	15	N.D.	N.D.
Gnotobiotic rats	7	5.57 $\pm$ 3.37	4.40 $\pm$ 1.97
	15	6.63 $\pm$ 3.97	4.80 $\pm$ 2.09

Values are means  $\pm$  s.d. (n = 3) 7 and 15 h after oral administration of ginsenoside Rb<sub>1</sub>. N.D., not detected.

Table 3. Recovery of ginsenoside Rb<sub>1</sub>, and the compound K content of the intestinal tracts and cumulative faeces of germ-free and gnotobiotic rats 7 and 15 h after oral administration of ginsenoside Rb<sub>1</sub>.

		Germ-free rats		Gnotobiotic rats	
		7 h	15 h	7 h	15 h
Small intestine	Ginsenoside Rb <sub>1</sub>	13.5	2.9	N.D.	N.D.
	Compound K	N.D.	N.D.	5.6	6.7
Caecum	Ginsenoside Rb <sub>1</sub>	67.3	51.7	32.0	N.D.
	Compound K	N.D.	N.D.	9.3	19.2
Colon + rectum	Ginsenoside Rb <sub>1</sub>	4.5	7.4	3.5	N.D.
	Compound K	N.D.	N.D.	0.6	2.1
Faeces	Ginsenoside Rb <sub>1</sub>	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<sup>-1</sup> ginsenoside Rb<sub>1</sub> (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<sub>1</sub>. Compound K was detected at concentrations of 4.8 and 83.4 ng mL<sup>-1</sup> in the plasma of the gnotobiotic rats 7 and 15 h, respectively, after oral administration of ginsenoside Rb<sub>1</sub>. The concentration of compound K in the plasma of gnotobiotic 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 gnotobiotic rats after oral administration of ginsenoside Rb<sub>1</sub>, but that no compound K was generated by the germ-free rats.

In conclusion, ginsenoside Rb<sub>1</sub>-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<sub>1</sub> to rats, and this probably applies to man also. Orally administered ginsenoside Rb<sub>1</sub> 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<sub>2</sub>, Rb<sub>3</sub>, Rc and Rd, and ginsenoside Rb<sub>1</sub>. 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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