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Biotransformation of Ginseng Extract to Cytotoxic Compound K and Ginsenoside Rh_2 by Human Intestinal Bacteria

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Abstract – When saponin extracts of dried ginseng and red ginseng were anaerobically incubated with human intestinal microflora, these extracts were metabolized to compound K and ginsenoside Rh_2, respectively. However, when these extracts were incubated with commercial lactic acid bacteria, these did not metabolize these ginsenosides to compound K or ginsenoside Rh_2. Among some intestinal bacteria isolated from human feces, *Bacteroides* C-35 and C-36 transformed these saponin extracts to compound K and ginsenoside Rh_2, respectively. These bacteria also transformed water extracts of dried ginseng and red ginseng to compound K and ginsenoside Rh_2, respectively, similarly with that of the saponin extracts. Among transformed ginsenosides, compound K and 20(*S*)-ginsenoside Rh_2 exhibited the most potent cyotoxicity against tumor cells.
Keywords – ginseng, intestinal bacteria, transformation, compound K, ginsenoside Rh_2, cytotoxicity.

Introduction

Ginseng (the root of *Panax ginseng* C.A. Meyer, Araliaceae) is frequently used as a crude substance taken orally in Asian countries as a traditional medicine. The major components of ginseng are ginsenosides, which contain glycosides with a dammarane skeleton (Tanaka *et al.*, 1972). These ginsenosides have been reported to show various biological activities including anti-inflammatory activity (Wu *et al.*, 1992) and anti-tumor effects (inhibition of tumor-induced angiogenesis and the prevention of tumor invasion and metastasis) (Mochizuki *et al.*, 1995; Sato *et al.*, 1994). To explain these pharmacological actions, it is thought that ginseng saponins are metabolized by human intestinal microflora after being taken orally (Kanaoka *et al.*, 1992; Kanaoka *et al.*, 1994; Karikura *et al.*, 1991; Akao *et al.*, 1998b). For example, ginsenosides Rb_1, Rb_2 and Rc are transformed to 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (compound K) by human intestinal bacteria (Akao *et al.*, 1998a; Hasegawa *et al.*, 1997; Bae *et al.*, 2000). This transformed compound K induces an anti-metastatic or anti-carcinogenic effect by blocking tumor invasion or preventing chromosomal aberration and tumorigenesis (Wakabayashi *et al.*, 1998; Lee *et al.*, 1999).

In addition, these ginsenosides Rb_1, Rb_2 and Rc were transformed to ginsenoside Rg_3 by the mild acid treatment such as stomach acid (Han *et al.*, 1982). Furthermore, this ginsenoside Rg_3 is a characteristic component of red ginseng, steamed ginseng (Kitagawa *et al.*). We reported that the ginsenoside Rg_3 transformed to ginsenoside Rh_2 by human intestinal bacteria (Bae *et al.*, 2002). This transformed ginsenoside Rh_2 showed more potent cytotoxic activity than ginsenoside Rg_3 or ginsenoside Rc. However, the biotransformation of ginseng extracts treated with and without steaming to cytotoxic compound K and ginsenoside Rh_2 by intestinal bacteria or microflora have not been thoroughly studied.

Therefore, we biotransformed ginseng extract to compound K and ginsenoside Rh_2 by intestinal bacteria or microflora and measured the cytotoxicity of ginseng saponin metabolites with and without serum against several tumor cells.

Materials and Methods

Materials and bacterial strains – Sodium thioglycolate and ascorbic acid were purchased from Sigma Chem. Co. (U.S.A.). General anaerobic medium (GAM) was purchased from Nissui Pharmaceutical Co., Ltd., (Japan). Tryptic soy (TS) broth was purchased from Difco Co. (U.S.A.).

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The other chemicals were of analytical reagent grade. Eighteen lactic acid bacteria were purchased from Korean Cell Type Collection (Dajeon, Korea). Ginseng saponin extracts, compound K and ginsenoside Rh2 were prepared according to the previous method (Bae *et al.*, 2000 and 2002).

Screening of Ginsenosides-hydrolyzing Intestinal Bacteria from Human Intestinal Microflora – Fresh human feces (or commercial yogurts) were anaerobically diluted 10^3 to 10^7-fold. Two hundred microliters of the diluted fecal suspension were inoculated in BL agar plates. The plates were anaerobically incubated at 37°C for 72 hrs. Fifty intestinal bacteria were isolated from several plates and identified according to Bergey's manual. These isolated intestinal bacteria were cultured in 50 ml of tryptic soy broth containing 0.01% sodium thioglycolate and 0.1% ascorbic acid (TSTA), and then each cultured cell was collected at 3000 × g for 10 min and washed twice with saline. The ginsenosides-hydrolyzing activities of these collected cells were measured according to the assay method below.

Assay of Metabolized Ginsenosides by Intestinal Microflora of Human – The reaction mixture containing 100 μl of each ginseng saponin extract (or ginseng extract) in various concentrations and 100 μl of fecal suspension (or bacterial suspension cultured in TSTA broth) was incubated for 20 hrs at 37°C. The reaction mixture was extracted with BuOH, evaporated and assayed by TLC: TLC plates, silica gel $60F_{254}$ (Merck Co., USA); developing solvent, $CHCl_3$-MeOH-H_2O (65:35:10 v/v, lower phase). The plates were stained by spraying with MeOH-H_2SO_4 (95:5 v/v), followed by heating. The stained TLCs were then analyzed by a TLC scanner (Shimadzu model CS-9301PC, Japan).

Each isolated bacterium was cultured in 50 ml TSTA broth and collected at 3000 × g for 10 min. Each collected bacterial pellet was suspended in 50 mM phosphate buffer and used as a bacterial solution.

Time Course of the Metabolism of Ginseng Saponin by Intestinal Microflora and Bacteria of Human – Ginse nosides metabolizing activity was measured as follows. 2 ml of lactic acid bacterial suspension (wet weight, 100 mg/ml) were added to 8 ml of anaerobic diluted medium containing 1% each ginseng water extract and then was incubated at 37°C for 24 hrs, and an aliquot (0.5 ml) of the reaction mixture was periodically extracted twice with 1 ml of BuOH. The BuOH fraction was analyzed by TLC. Ginsenosides and their metabolites were identified and assayed by authentic compounds isolated according to previously reported methods (Bae *et al.*, 2000 and 2002).

The lactic acid bacteria were cultured in 500 ml of TS broth and centrifuged at 10000 × g for 30 min and washed with the anaerobic dilution medium. The fecal or bacterial precipitates (250 mg) were resuspended in 1.75 ml of anaerobic dilution medium.

***In Vitro* Cytotoxicity Assay** - The *in vitro* cytotoxicity was evaluated against P388 (mouse lymphoid neoplasma cell line), A549 (human lung carcimoma), HepG2 (human liver hepatoblastoma) and HeLa (human cervix uterine adenocarcinoma) cells by MTT [3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium 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: 1.5×10^4 cells/well) into 96-well microtiter plate. P338, A549, HepG2 and HeLa cells were cultured for 24 h in media with or without fatal bovine serum (FBS) and treated with the samples. These cells were exposed to the test compounds for 48 at 37°C. 50 μl of MTT solution (2 mg/ml in PBS) was added to each well and the plates were incubated for 1 h. After aspiration of the medium, DMSO (100 μl) was added to solubilize the MTT-formazan product. The plates were read on a microplate reader (540 nm). The 50% cytotoxic concentration (EC_{50}) of tumor cell growth was defined compared with the control cell culture.

Results and Discussion

When the saponin fraction of dried ginseng was incubated with human intestinal bacteria, most of fecal specimens transformed these compounds into compound K. Therefore, fifty intestinal bacteria were isolated from human feces and their transforming activities of ginsenosides to compound K were measured (Table 1). Among fifty intestinal bacteria, fifteen bacteria transformed ginsenoside Rd, eight bacteria produced ginsenoside F_2, and two bacteria C-35 and C-36 transformed ginsenosides to compound K. C36 transformed more potently ginseng saponin to compound K than C-35. C-35 and C-36 all,

Table 1. Distribution of intestinal bacteria to transform ginseng saponin into compound K

	Number of transforming bacteria			
	Ginsenoside Rd	Ginsenoside F_2	Compound K	Ginsenoside Rh_2
Intestinal bacteria (50)	15	8	2	0
Commercial probiotics (18)	0	0	0	0

which were gram-negative and anaerobes, were identified to be *Bacteroides* species by the identification according to the Bergey's manual. However, commercial lactic acid bacteria, *Lactobacillus acidophilus, Streptococcus thermophilus* and *Lactobacillus casei*, were not found to transform the ginsenosides to compound K. The saponin fraction of dried ginseng in various concentrations was incubated with *Bacteroides* C-36 for 24 h, and then amounts of the compound K and ginsenoside Rh_2 were measured (Fig. 1A). The compound K was dose-dependently produced from ginseng saponin extract. However, the productivity of compound K was decreased at more than 1% of ginseng saponins extract. And ginsenoside Rh_2 was not produced from the saponin extract of dried ginseng. The saponin extract of red ginseng in various concentrations was incubated with *Bacteroides* C-36 for 24 h, and then amounts of the compound K and ginsenoside Rh_2 were also measured (Fig. 1B). The ginsenoside Rh_2 was dose-dependently produced from ginseng saponin extract. However, the productivity of ginsenoside Rh_2 was decreased at more than 0.1% of ginseng saponins extract. This may be due to the antibacterial activity of ginseng extracts (Table 2). And compound K was found to be weakly produced from the saponin extract of steamed ginseng. The water extract of dried ginseng in various concentrations was also incubated with *Bacteroides* C-36 for 24 h, and then amounts of the compound K and ginsenoside Rh2 were measured (Fig. 2A). The compound K was dose-dependently produced from ginseng saponin extract. However, the productivity of the transformed compound K was decreased at more than 0.06% of ginseng saponins extract. And ginsenoside Rh2 was not found to be produced from the saponin extract of dried ginseng.

Table 2. Effect of ginseng extract on the growth of ginseng saponin-transforming *Bacteroides* sp

	MIC (mg/ml)	
	Without transformation	With transformation
Bacteroides C-35	250	50
Bacteroides C-36	250	50

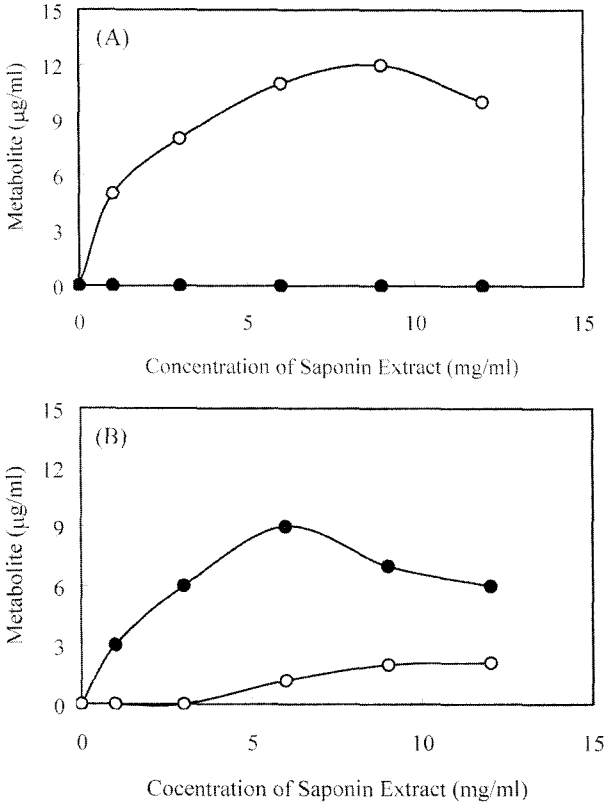


Fig. 1. Productivity of compound K and ginsenoside Rh_2 from ginseng saponin extracts of dried ginseng (A) and red ginseng (B) by *Bacteroides* C-36 isolated from human intestinal microflora. The bacterial suspension was prepared according to MATERIALS AND METHODS. ○, compound K; ●, ginsenoside Rh_2.

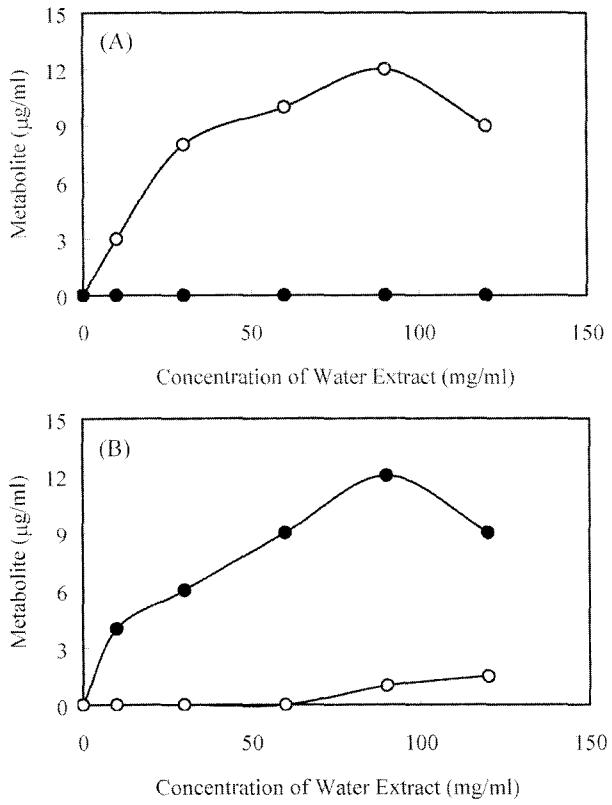


Fig. 2. Productivity of compound K and ginsenoside Rh_2 from ginseng water extracts of dried ginseng (A) and red ginseng (B) by *Bacteroides* C-36 isolated from human intestinal microflora. The bacterial suspension was prepared according to MATERIALS AND METHODS. ○, compound K; ●, ginsenoside Rh_2.

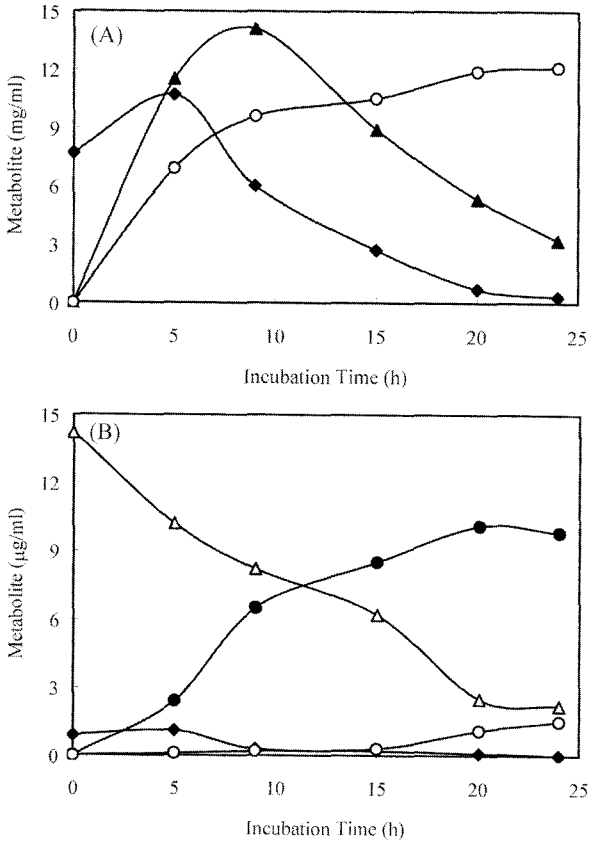


Fig. 3. Time course of biotransformation of ginseng extracts of dried ginseng (A) and red ginseng (B) by human intestinal microflora. Human fecal suspension was prepared and their metabolites were assayed according to MATERIALS AND METHODS. ○, compound K; ●, ginsenoside Rh_2; ◆, ginsenoside Rd; △, ginsenoside Rg_3. ▲, ginsenoside F_2.

The water extract of red ginseng in various concentrations was also incubated with *Bacteroides* C-36 for 24 h, and then amounts of the compound K and ginsenoside Rh2 were measured (Fig. 2B). The ginsenoside Rh_2 was dose-dependently produced from ginseng saponin extract. However, the productivity of ginsenoside Rh_2 was decreased at more than 1% of ginseng saponins extract. And compound K was found to be weakly produced from the saponin extract of red ginseng.

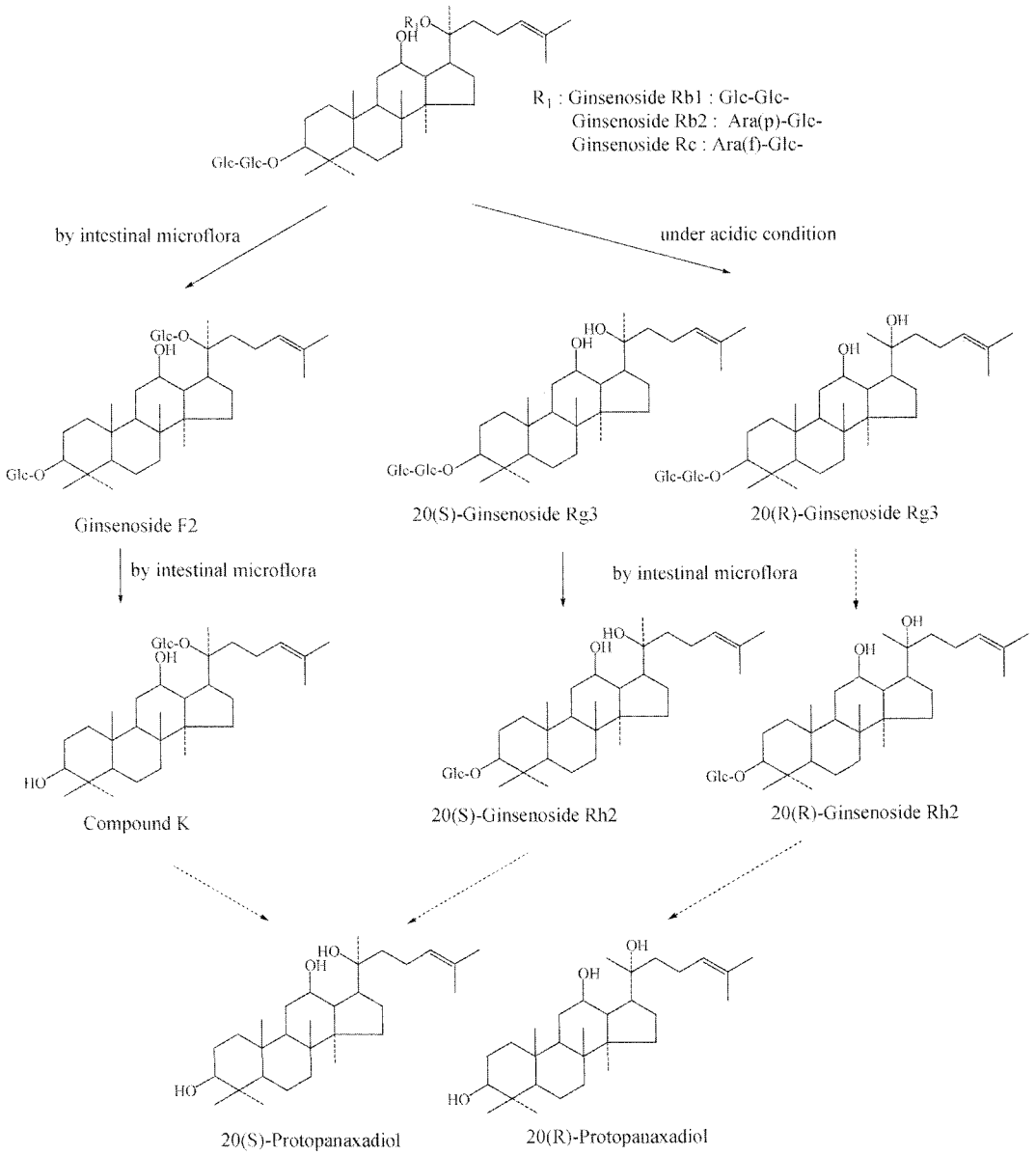
To understand the metabolic pathway of ginsenosides by human intestinal microflora, *Bacteroides* C-36 was incubated with saponin extracts of dried ginseng and metabolites were analyzed. First ginsenoside Rd was increased, and then ginsenoside F_2 and compound K were produced (Fig. 3A). A main metabolite was compound K after 24 h incubation. When the C-36 was incubated with saponin extract of red ginseng, first ginsenoside Rh_2 was increased instead of ginsenoside Rg_3 (Fig. 3B), indicating that a main metabolite was ginsenoside Rh_2 after 24 h incubation.

To evaluate the cytotoxicity of the saponin extract of ginseng treated by human intestinal microflora, the cytotoxicity of ginseng extracts and ginsenosides with and without transformation were measured. Ginseng saponin extract was not shown to exhibit cytotoxic activity against tumor cell lines, however, the transformed ginseng saponin extract exhibited cytotoxic activity. We also measured the cytotoxicity of ginsenosides isolated from dried ginseng, red ginseng and their transformed ginsengs. The compound K and ginsenoside Rh_2 isolated from the transformed ginsengs exhibited the most potent cytotoxicity. These

Table 3. The cytotoxicity of some ginsenosides against several tumor cells

	EC_{50} (μM)							
	With FBS				Without FBS			
	A549	P388	HeLa	HepG2	A549	P388	HeLa	HepG2
Dried ginseng saponin extract (GSE)	>100	>100	-[a]	-	-	-	-	-
Red GSE (RGSE)	>100	>100	-	-	-	-	-	-
GSE metabolite	>100	98	-	-				
RGSE metabolite	>100	95	-	-	-	-	-	-
Ginsenoside Rb1	>50	>50	>50	>50	>50	-	>50	>50
Ginsenoside Rb2	>50	>50	>50	>50	>50	-	>50	>50
Ginsenoside Rc	>50	>50	>50	>50	>50	-	>50	>50
Ginsenoside Rd	>50	>50	>50	>50	>50	-	>50	>50
20(S)-Ginsenoside Rg3	>50	>50	>50	>50	28.9	-	>50	>50
20(R)-Ginsenoside Rg3	>50	>50	>50	>50	>50	-	>50	>50
Compound K	27.9	31.6	27.1	28.8	0.1	-	0.1	0.6
20(S)-Ginsenoside Rh2	>50	37.6	>50	>50	3.4	-	0.7	7.2
20(R)-Ginsenoside Rh2	>50	>50	>50	>50	>50	-	>50	>50
Adriamycin	10.6	1.9	4.1	2.2	0.9	0.6	0.7	3.8

Each ginsenoside was treated for 48 h in the media with and without fetal bovine serum. [a] not detected.
ED_{50} represents 50% cytotoxic concentration compared to viability of control.



Scheme 1. Proposed metabolic pathway of protopanaxadiol glycosides from ginseng.

ginsenosides showed more potent cytotoxicity in the media with and without FBS: EC_{50} of compound K were 27.1-31.6 μM and 0.1-0.6 μM, and those of 20(*S*)-ginsenoside Rh2 were 37.5-65 μM and 0.7-7.1 μM, respectively.

Ginseng, which contains ginsenoside Rb_1, Rb_2 and Rc as its main components, and red ginseng, which contains ginsenoside Rg_3 and Rg_1, are frequently used as a crude drug taken orally in Asia. These components were transformed to compound K or ginsenoside Rh_2 by intestinal microflora to explain their anti-metastatic and anti-carcinogenic activities *in vivo*. When water extract of dried ginseng was incubated with human intestinal microflora, it was mainly transformed to compound K via ginsenoside F_2 according to the intestinal bacteria (Scheme 1). This result supported the results reported previously by Akao *et al.* (1998) and Hasegawa *et al.* (1997). When water extract of red ginseng was incubated with human intestinal microflora, it was mainly transformed to ginsenoside Rh_2 by intestinal microflora (Scheme 1). This result supports the results reported previously by Bae *et al.* (2002). Therefore, if ginseng extract was transformed to compound K or ginsenoside Rh_2 in human intestine, antimetastatic and/or cytotoxic activities of ginseng should be increased. Most fecal specimens of human beings exhibited the metabolic

activity of ginsenosides to compound K or ginsenoside Rh_2, but some specimens did not transform it. Furthermore, when a high dose of ginseng extracts (1g/kg) was orally administered to rats, ginsenosides was not found to be transformed sufficiently to compound K or ginsenoside Rh_2 by human intestinal bacteria (Data not shown). Therefore, antitumor activity of fermented ginseng may be more effective than that of dried ginseng. Based on these findings, we insist that ginseng saponins may be prodrugs, which can be transformed to active compound K or ginsenoside Rh_2 by intestinal microflora.

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