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Transformation of Ginsenosides to Compound K (IH-901) by Lactic Acid Bacteria of Human Intestine

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Abstract When ginsenosides Rb1, Rb2, and Rc were anaerobically incubated with commercial and human intestinal lactic acid bacteria, most commercial lactic acid bacteria did not metabolize these ginsenosides to compound K. However, lactic acid bacteria, *B. minimum* KK-1, *Bifidobacterium cholerium* KK-2, and *B. cuniculi* K-513, isolated from human intestinal microflora transformed these ginsensosides to compound K. When the bacterial mixtures of commercial lactic acid bacteria were incubated with these ginsenosides, these compounds were not transformed to compound K. However, when *Bifidobacterium* KK-1 and KK-2 were mixed, these ginsenosides were synergistically transformed to compound K. When water extract of ginseng was incubated with these mixed bifidobacteria, compound K was potently produced. Therefore, it is suggested that, if ginseng with these mixed bifidobacteria is fermented, compound K-enforced ginseng materials could be produced that show cytotoxicity against tumor cell lines.

Key words: Ginsenosides, lactic acid bacteria, compound K, cytotoxicity

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 [9, 13]. These ginsenosides have been reported to show various biological activities including anti-inflammatory activity [17] and antitumor effects (inhibition of tumor-induced angiogenesis and the prevention of tumor invasion and metastasis) [15, 16]. To explain these pharmacological actions, it has been suggested that ginseng saponins must be metabolized by human intestinal microflora after being taken orally [1, 2, 7, 8]. For example, ginsenosides Rb1, Rb2, and Rc are transformed to 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (IH-901, compound K) by human intestinal bacteria [6]. This transformed IH-901 induces an antimetastatic or anticarcinogenic effect by blocking tumor invasion or preventing chromosomal aberration and tumorigenesis [11, 16]. However, all human fecal specimens could not metabolize these ginsenosides to the compound K. Therefore, to prepare the compound K-containing fermented ginseng materials, the lactic acid bacteria capable of metabolizing ginsenosides to the compound K were investigated.

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Materials and Methods

Materials and Bacterial Strains

Sodium thioglycolate and ascorbic acid were purchased from Sigma Chem. Co. (St. Louis, U.S.A.). General anaerobic medium (GAM) and glucose blood liver agar (BL) were purchased from Nissui Pharmaceutical Co., Ltd., (Tokyo, Japan). Tryptic soy broth was purchased from Difco Co. (Detroit, U.S.A.). The other chemicals were of analytical reagent grade.

Bifidobacterium longum KCTC3215 and *B. animalis* KCTC3126 were purchased from KCTC. *B. infantis* JCM7007, *B. adolescentis* JCM1275, *B. bifidum* JCM1254, and *B. breve* JCM1192 were purchased from JCM. *B. minimum* KK-1, *B. cholerium* KK-2, *B. bifidum* K-105, *B. breve* K-111, *B. catenulatum* K-309, *B. magnum* K-321, and *B. cuniculi* K-513 were isolated from human intestinal microflora. Some commercial lactic acid bacteria, *Lactobacillus bulgaricus*, *L. brevis*, and *Streptococcus thermophilus*, were isolated from commercial yogurts according to the previous methods [10, 12].

Ginsenosides Rb1, Rb2, and Rc were isolated from ginseng according to the previously described method [3]. Compound K was prepared by *Fusobacterium* K-60 biotransformation of ginsenoside Rb1 and silica gel column chromatographgy [3]. 20(*S*)-Protopanaxadiol was prepared by the method previously reported [4].

Screening of Ginsenosides-Hydrolyzing Lactic Acid 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 h. Two hundred lactic acid bacteria were isolated from several plates and identified according to Bergey's manual. These isolated lactic acid 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 cells were collected at 3,000 rpm 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 Metabolic Activity of Ginsenosides by Lactic Acid Bacteria

The reaction mixture (5 ml) containing 0.5 mg each ginsenoside and 250 mg fecal suspension (or bacterial suspension cultured in TSTA broth) was incubated for 20 h at 37°C. The reaction mixture was extracted with BuOH, evaporated, and assayed by TLC: TLC plates, silica gel $60F_{254}$ (Merck Co., Whitehouse station, U.S.A.); 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, Tokyo, Japan).

Each isolated bacterium was cultured in 50 ml TSTA broth and collected at 3,000 rpm for 30 min. Each collected bacterial pellet was suspended in 50 mM phosphate buffer and used as a crude enzyme solution.

Time Course of the Metabolism of Ginsenosides by Lactic Acid Bacteria

Ginsenosides metabolizing activity was measured as follows. Five milliliters of lactic acid bacterial suspension (500 mg) were added to 5 ml of anaerobic diluted medium containing ginsenoside Rb1 (or ginsenoside Rb2 or ginsenoside Rc) (final concentration in reaction mixture, 120 μM) or ginseng extract (final concentration, 0.2 mg/ml), and the mixture was incubated at 37°C for 24 h, 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 the previously described methods [3].

The isolated lactic acid bacteria were cultured in 500 ml TSTA broth, centrifuged at 10,000 rpm for 30 min, and washed with the anaerobic dilution medium. The fecal and bacterial precipitates (500 mg) were resuspended in 5 ml of anaerobic dilution medium.

***In Vitro* Cytotoxicity Assay**

In vitro cytotoxicity was tested against L1210 (mouse lymphocytic leukemia cell line), P388 (mouse lymphoid neoplasma cell line), A549 (human lung carcinoma), and Me180 (human cervix uterine carcinoma) by MTT [3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to the method of Carmichael *et al.* [5]. Each cultured cell line was harvested, counted, and inoculated at the appropriate concentrations (180 μl volume: 4×10^4 cells/well for P388 and L1210; 3×10^4 cells/well for A549 and Me180) into 96-well microtiter plates. P338 and L1210 cell lines were then cultured for 2 h and A549 and Me180 cell lines for 24 h. These cells were exposed to the test compounds for 2 days at 37°C. Fifty ml of MTT

Table 1. Transformed level of ginsenosides to compound K by lactic acid bacteria.

Microbe	Compound K (μM)		
	Ginsenoside R_{b1}	Ginsenoside R_{b2}	Ginsenoside R_c
B. longum KCTC3215	–	–	–
B. infantis JCM7007	–	–	–
B. adolescentis JCM1275	–	–	–
B. longum JCM1217	–	–	–
B. animalis KCTC3126	–	–	–
B. bifidum JCM1254	–	–	–
B. breve JCM1192	–	–	–
Lactobacillus brevis	–	–	–
L. bulgaricus	–	–	–
Streptococcus thermophilus	–	–	–
B. minimum KK-1	4.89	5.88	2.15
B. cholerium KK-2	0.68	1.12	0.98
B. bifidum K-105	–	–	–
B. breve K-111	–	–	–
B. catenulatum K-309	–	–	–
B. magnum K-321	–	–	–
B. cuniculi K-513	0.21	0.45	0.79
B. minimum KK-1 and *B. cholerium* KK-1	41.5	33.5	31.40
B. minimum KK-1 and *B. cuniculi* K-513	11.33	7.87	6.56
B. cholerium KK-2 and *B. cuniculi* K-513	2.0	1.10	1.10
Human intestinal microflora	72	68	59

The reaction mixture (5 ml) contained 0.5 mg of each ginsenoside and 250 mg of microbial suspension.

Table 2. Transforming activity of ginseng extract to compound K by mixed bifidobacteria suspension.

Microbe	Compound K (μM)
B. minimum KK-1	11.1
B. cholerium KK-2	5.9
B. minimum KK-1 and *B. cholerium* KK-2	77
Human intestinal microflora	120

The reaction mixture (5 ml) contained 2.5 mg of ginseng extract and 250 mg of microbial suspension.

Table 3. Cytotoxicity of ginseng and fermented ginseng against tumor cell lines.

Material	IC_{50} (μM)			
	L1210	P388	Mel80	A549
Ginseng extract	>100	>100	>100	>100
Fermented ginseng extract	27	54	>100	86
Ginsenoside Rb1	>100	>100	>100	>100
Ginsenoside Rb2	>100	>100	>100	>100
Ginsenoside Rc	>100	>100	>100	>100
Compound K	24	33	33	34
Cisplatin	3.87	5.49	17.8	27.9

solution (2 mg/ml in PBS) was added to each well and the plates were incubated for 4 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% inhibitory concentration (IC_{50}) of tumor cell growth was defined compared with the control cell culture.

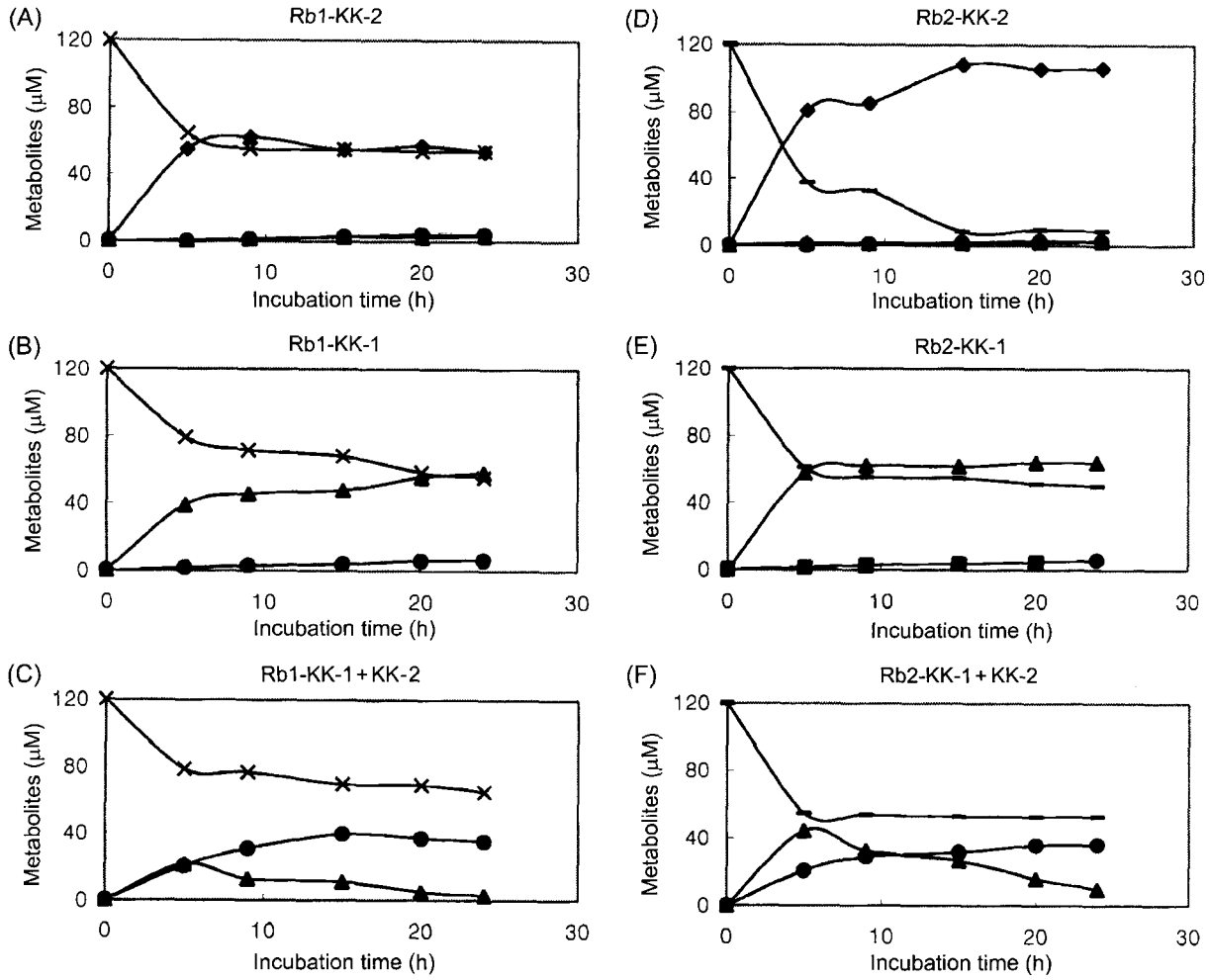

Fig. 1. Time course of ginsenoside Rb1 and Rb2 by lactic acid bacteria isolated from human intestinal microflora.
The intestinal bacterial suspension was prepared and their metabolites were assayed according to Materials and Methods. (A) Gins enoside Rb1 (120 μM) was treated with KK-2 (500 mg), (B) ginsenoside Rb1 (120 μM) was treated with KK-1 (500 mg), (C) Ginsenoside Rb1 (120 μM) was treated with KK-2 (250 mg) and KK-1 (250 mg), (D) Ginsenoside Rb2 (120 μM) was treated with KK-2 (500 mg), (E) ginsenoside Rb2 (120 μM) was treated with KK-1 (500 mg), and (F) Ginsenoside Rb2 (120 μM) was treated with KK-2 (250 mg) and KK-1 (250 mg). ×, ginsenoside Rb1; –, ginsenoside Rb2; ◆, ginsenoside Rd; ▲, ginsenoside F2; ●, compound K (IH901).

RESULTS AND DISCUSSION

When ginsenosides Rb1, Rb2, or Rc were incubated with human intestinal bacteria, most fecal specimens transformed these compounds to the compound K or protopanaxadiol. Therefore, lactic acid bacteria from human feces were isolated and their transforming activities of ginsenosides to the compound K were measured (Table 1). Most lactic acid bacteria isolated from human feces did not transform these ginsenosides to the compound K. And the commercial lactic acid bacteria, *Lactobacillus acidophilus*, *Streptococcus thermophilus*, and *Lactobacillus casei*, could not transform the ginsenosides to compound K. However, *B. minimum* KK-1, *B. cholerium* KK-2, and *B. cuniculi* K-513 isolated from human intestinal microflora only transformed these ginsenosides to compound K. Particularly, *B. minimum* KK-1 exhibited the most potent transforming activity. However, bifidobacteria isolated from human fecal microflora transformed these ginsenosides to the compound K less strongly than the human fecal suspension. Therefore, lactic acid bacteria isolated from human intestinal microflora was mixed, and the activity of transforming ginsenosides to compound K was measured. The mixed bifidobacteria synergistically transformed these ginsenosides to compound K. However, the bifidobacteria formulation mixed without *B. minimum* KK-1 was not synergic. Particulary, *B. cholerium* KK-2 with *B. minimum* KK-1 appeared to have the most synergic transforming activity. However, the mixed suspension of commercial lactic acid bacteria did not transform the ginsenosides to the compound K.

The transforming activity of the ginseng extract to the compound K by isolated lactic acid bacteria and mixed bacteria was also measured (Table 2). *B. minimum* KK-1 exhibited the most potent transforming activity. The mixed *Bifidobacterial* suspension with *B. minimum* KK-1 transformed synergistically ginseng extract to the compound K.

We also measured the cytotoxic activity of the transformed ginseng extract by *B. cholerium* KK-2 with *B. minimum* KK-1 against tumor cell lines (Table 3). The transformed ginseng extract exhibited more potent cytotoxic activity against tumor cell lines than the ginseng extract.

Ginseng, which contains ginsenosides Rb1, Rb2, and Rc as its main components, is frequently used as a crude drug taken orally in Asia. These components should be transformed to the compound K (IH-901) by intestinal microflora, which explains their antimetastatic and anticarcinogenic activities *in vivo*. Akao *et al.* and Hasegawa *et al.* reported that the metabolic pathway of these ginsenosides proceeded to compound K (IH-901) via ginsenoside F2 [2, 6]. We also observed that the ginseng extract was mainly transformed to the compound K (IH-901) or 20(*S*)-protopanaxadiol via ginsenoside F2 by the intestinal bacteria (Fig. 1), when it was incubated with human intestinal microflora. *Bifidobacteria* isolated from human intestinal microflora also transformed these ginseng extracts to the compound K via ginsenoside F2. The metabolic pathway of the ginseng water extract by bifidobacteria was similar to that by intestinal microflora.

Most fecal specimens of human beings were capable of metabolic conversion of ginsenosides to the compound K, but some specimens could not transform these ginsenosides to the compound K. Furthermore, when a high dose of ginseng extract (1 g/kg) was administered to rats (data not shown), the ginsenosides could not be transformed sufficiently to the compound K. Therefore, if ginseng extract was treated with lactic acid bacteria, antimetastatic or cytotoxic activities of ginseng should be increased. Most isolated intestinal lactic acid bacteria and commercial lactic acid bacteria hydrolyze synthetic β-glucosidase substrate, PNG (data not shown). However, some intestinal lactic acid bacteria, not commercial bacteria, could catalyze the conversion of ginsenosides to the compound K. These mixed lactic acid bacteria also potently transformed ginsenosides to compound K. These results suggest that lactic acid bacteria should produce many kinds of glucosidases.

When the cytotoxic activity of ginseng extract and fermented ginseng extract against tumor cell lines was measured, ginseng extract did not exhibit the cytotoxic activity against tumor cell lines. However, the fermented ginseng extract exhibited a potent cytotoxic activity against tumor cell lines. These results suggest that, if ginseng extract were orally administered, ginsenosides Rb1, Rb2, and Rc could be transformed to compound K (IH 901) in human intestine [3, 6] and the transformed compound, compound K, could exhibit the pharmacological activities, such as cytotoxicity againt tumor cell lines (Fig. 3). These

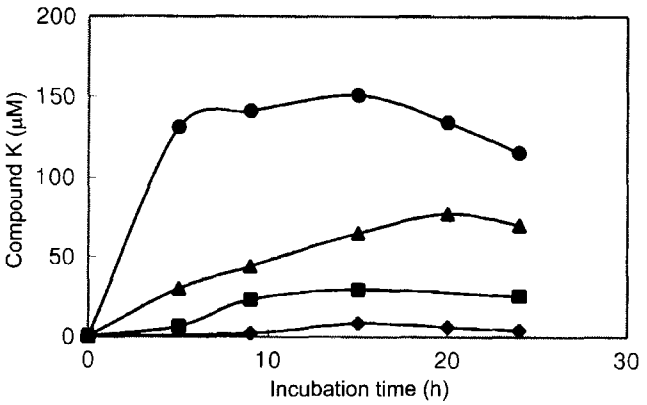

Fig. 2. Time course of biotransformation of ginseng extract to compound K by lactic acid bacteria and human intestinal microflora.
Human fecal suspension was prepared and their metabolites were assayed according to Materials and Methods. ◆, Ginseng extract (200 μg/ml) was treated with KK-2 (100 mg/ml); ▲, ginseng extract (200 μg/ml) was treated with KK-1 (100 mg/ml); ▲, ginseng extract (200 μg/ml) was treated with KK-2 (50 mg/ml) and KK-1 (50 mg/ml); ●, ginseng extract was treated with human intestinal microflora (100 mg/ml).

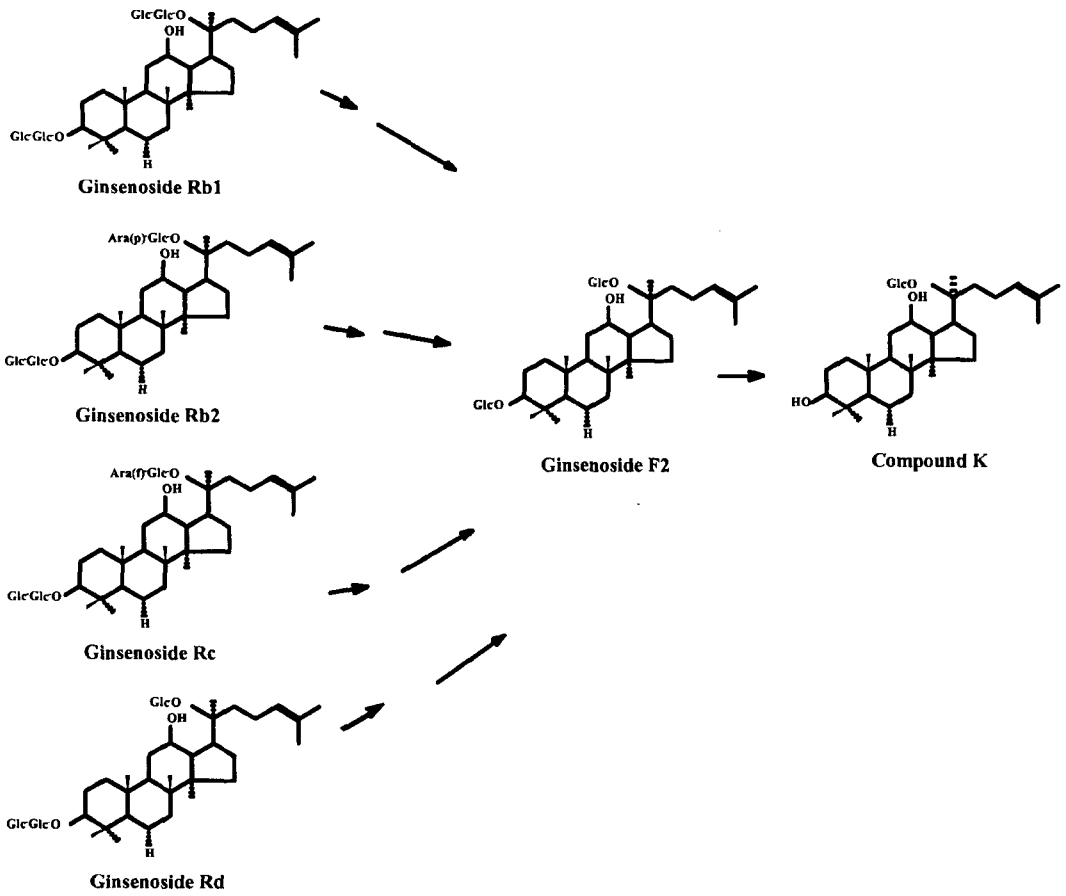

Fig. 3. Proposed metabolic pathway of protopanaxadiol glycosides from ginseng.

results suggest that the natural glycosides, ginsenosides Rb1, Rb2, and Rc, are prodrugs, which can be transformed to active compounds by intestinal microflora. Finally, we believe that if ginseng with these mixed bifidobacteria is fermented, the compound K-enforced ginseng materials could be produced and will be useful to prevent tumors.

REFERENCES

1. Akao, T., M. Kanaoka, and K. Kobashi. 1998. Appearance of compound K, a major metabolite of ginsenoside Rb1 by intestinal bacteria, in rat plasma after oral administration measurement of compound K by enzyme immunoassay. *Biol. Pharm. Bull.* **21:** 245–249.
2. Akao, T., H. Kida, M. Kanaoka, M. Hattori, and K. Kobashi. 1998. Intestinal bacterial hydrolysis is required for the appearance of compound K in rat plasma after oral administration of ginsenoside Rb1 from Panax ginseng. *J. Pharm. Pharmacol.* **50:** 1155–1160.
3. Bae, E.-A., S.-Y. Park, and D.-H. Kim. 2000. Constitutive β-glucosidases hydrolyzing ginsenoside Rb1 and Rb2 from human intestinal bacteria. *Biol. Pharm. Bull.* **23:** 1481–1485.
4. Bae, E. A., M. J. Han, M.-K. Choo, S.-Y. Park, and D.-H. Kim. 2002. Metabolism of 20(*S*)- and 20(*R*)-ginsenoside Rg3 by human intestinal bacteria and its relation to *in vitro* biological activities. *Biol. Pharm. Bull.* **25:** 58–63.
5. Carmichael, J., W. G. DeGreff, A. F. Gazdar, J. D. Minna, and J. B. Mitchell. 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res.* **47:** 936–940.
6. Hasegawa, H., J.-W. Sung, and Y. Benno. 1997. Role of human intestinal *Prevotella oris* in hydrolyzing *Ginseng saponis*. *Planta Medica* **63:** 436–440.
7. Kanaoka, M., H. Kato, F. Shimada, and S. Yano. 1992. Studies on the enzyme immunoassay of bioactive constituents contained in oriental medicinal drugs. VI. Enzyme immunoassay of ginsenoside Rb1 from *Panax ginseng*. *Chem. Pharm. Bull.* **40:** 314–317.
8. Kanaoka, M., T. Akao, and K. Kobashi. 1994. Metabolism of ginseng saponins, ginsenosides, by human intestinal bacteria. *J. Tradit. Med.* **11:** 241–245.
9. Karikura, M., T. Miyaze, H. Tanizawa, T. Taniyzma, and Y. Takino. 1991. Studies on absorption, distribution, excretion and metabolism of ginseng saponins. VII. Comparison of the decomposition modes of ginsenoside Rb1 and Rb2 in the digestive tract of rats. *Chem. Pharm. Bull.* **38:** 2357–2361.

10. Kim, D.-W., H.-J. Hwang, and K.-H. Park. 2001. Physiological responses of oxygen-tolerant anaerobic *Bifidobacterium longum* under oxygen. *J. Microbiol. Biotechnol.* **11:** 443–451.
11. Lee, S. J., J. H. Sung, S. J. Lee, C. K. Moon, and B. H. Lee. 1999. Antitumor activity of a novel ginseng saponin metabolite in human pulmonary adenocarcinoma cells resistant to cisplatin. *Cancer Lett.* **144:** 39–43.
12. Lee, K.-H., G. S. Moon, J.-Y. An, H. J. Lee, H. C. Chang, D. K. Chung, J. H. Lee, and J. K. Kim. 2002. Isolation of nisin-producing *Lactobacillus lactis* strain from Kimchi and characterization of its *nisZ* gene. *J. Microbiol. Biotechnol.* **12:** 389–397.
13. Mochizuki, M., C. Y. Yoo, K. Matsuzawa, K. Sato, I. Saiki, S. Tono-oka, K. Samukawa, and I. Azuma. 1995. Inhibitory effect of tumor metastasis in mice by saponins, ginsenoside Rb2, 20(*R*)- and 20(*S*)-ginsenoside Rg3, of Red ginseng. *Biol. Pharm. Bull.* **18:** 1197–1202.
14. Tanaka, N., O. Tanaka, and S. Shibata. 1972. Chemical studies on the oriental plant drugs. XXVIII. Saponins and sapogenins of ginseng; Stereochemistry of sapogenin of ginsenoside Rb1, Rb2 and Rc. *Chem. Pharm. Bull.* **20:** 1212–1216.
15. Sato, K., M. Mochizuki, I. Saiki, Y. C. Yoo, K. Samukawa, and I. Azuma. 1994. Inhibition of tumor angiogenesis and metastasis by a saponin of *Panax ginseng* - ginsenoside Rb2. *Biol. Pharm. Bull.* **17:** 635–639.
16. Wakabayashi, C., H. Hasegawa, J. Murata, and I. Saiki. 1998. *In vivo* antimetastatic action of ginseng protopanaxadiol saponins is based on their intestinal bacterial metabolites after oral administration. *Oncol. Res.* **9:** 411–417.
17. Wu, J. Y., B. H. Gardner, C. I. Murphy, J. R. Seals, C. R. Kensil, J. Recchia, G. A. Beltz, G. W. Newman, and M. J. Newman. 1992. Saponin adjuvant enhancement of antigen-specific immune responses to an experimental HIV-1 vaccine. *J. Immunol.* **148:** 1519–1525.