Transformation of Ginsenosides Rb2 and Rc from *Panax ginseng* by Food Microorganisms

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Protopanaxadiol ginsenosides Rb2 and Rc were transformed using cell-free extracts from various edible food microorganisms and then analyzed by TLC and HPLC. Rb2 and Rc were transformed into compound K via Rd and F2 by *Bifidobacterium* sp. Int57 and *Bifidobacterium* sp. SJ32. *Lactobacillus delbrueckii* transformed Rb2 and Rc into ginsenoside Rh2. *Bifidobacterium* sp. SH5 transformed Rb2 and Rc into F2. *Aspergillus niger* transformed Rb2 into compound K via compound O and compound Y, whereas it transformed Rc into compound K via Mc. Taken together, these processes would allow a specific bioconversion process to obtain specific ginsenosides using an appropriate combination of ginsenoside substrates and specific microbial enzymes.

Key words ginsenoside; transformation; food microorganism; microbial cell extract

Ginseng (the root of *Panax ginseng* C. A. MEYER; family Araliaceae) is used worldwide for the prevention of various diseases. Ginseng saponins (ginsenosides) are the principal components having pharmacological and biological activities, such as antidiabetic, and anti-tumor activities.^{1,2)} More than 30 different ginsenosides so far have been isolated and identified from ginseng saponins. Among them, the abbreviations of the ginsenosides analyzed in this study are shown in Table 1. The main ginsenosides are glycosides that contain an aglycone with a dammarane skeleton, and include protopanaxadiol-type saponins such as ginsenosides Rb1, Rb2, Rc and Rd, and protopanaxatriol-type saponins such as ginsenosides Re and Rg1. Among them, deglycosylated ginsenosides are known to be more readily absorbed into the bloodstream and to act as active compounds.³⁾ Intestinal microflora can transform ginsenosides into more active forms after ingestion of the ginsenosides.⁴⁾ Protopanaxadiol ginsenosides such as Rb1, Rb2, and Rc have previously been shown to be metabolized by human intestinal bacteria to their final derivative, compound K, with the degree of the transformation of the ginsenosides and production of compound K differing between *Eubacterium* sp., *Streptococcus* sp., and *Fusobacterium* K-60.⁵⁾ The efficiency of conversion and the transformation pathways may differ greatly due to the diversity of the resident microflora between individuals.⁵⁾ The above observations have lead to many attempts to produce transformed ginsenosides. Some researchers have attempted to prepare genuine prosapogenins or sapogenin by chemical

synthesis, mild acid hydrolysis, and alkaline cleavage.^{6,7)} However, these methods are associated with inevitable and undesirable side reactions, such as epimerization, hydration, and hydroxylation. These problems have lead to microbial conversion^{3,8–10)} and enzymatic conversion^{11,12)} to transform ginsenosides. Although several ginsenosides transformed by microorganisms were identified, investigations of the transformation pathway have been limited to only a few microorganisms. In addition, most of the microorganisms used for the transformation of ginsenosides in the previous studies were inedible. To rectify this, we instead used various microorganisms that have been safely used for foods consumption.

In this study we focused on the production of specific deglycosylated ginsenosides by using major protopanaxadiol ginsenosides Rb2 and Rc as substrates, along with enzyme preparations obtained from edible food microorganisms.

MATERIALS AND METHODS

Materials Standard Rc was purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Rb2, F2, and Rh2 were purchased from LKT Laboratories (St. Paul, MN, U.S.A.). Rd was purchased from BTGin (Chungnam, Korea), and compound K was donated from the Department of Pharmaceutical Science, Kyung Hee University (Seoul, Korea).

Microbial Strains and Growth Conditions *Bifidobacterium* sp. Int57, *Bifidobacterium bifidum* BGN4, *Bifidobac-*

Table 1. Abbreviations for Ginsenosides

Abbreviation	Full name
Rb2	3-O-[β-D-Glucopyranosyl-(1→2)-β-D-glucopyranosyl]-20-O-[α-L-arabinopyranosyl-(1→6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol
Rc	3-O-[β-D-Glucopyranosyl-(1→2)-β-D-glucopyranosyl]-20-O-[α-L-arabinofuranosyl-(1→6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol
Rd	3-O-[β-D-Glucopyranosyl-(1→2)-β-D-glucopyranosyl]-20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol
F2	3-O-β-D-Glucopyranosyl-20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol
Compound K	20-O-β-D-Glucopyranosyl-20(S)-protopanaxadiol
Rh2	3-O-β-D-Glucopyranosyl-20(S)-protopanaxadiol
Compound O	3-O-β-D-Glucopyranosyl-20-O-[α-L-arabinopyranosyl-(1→6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol
Compound Y	20-O-[α-L-Arabinopyranosyl-(1→6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol
Mc	20-O-[α-L-Arabinofuranosyl-(1→6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol

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terium sp. SJ32, and *Bifidobacterium* sp. SH5 were isolated as reported previously.¹³ *Lactobacillus delbrueckii* sp. *delbrueckii* KCTC 1047, *Aspergillus niger* KCTC 6906, and *A. usamii* var. *shirousamii* KCTC 6956 were purchased from the Korean Collection for Type Cultures (Daejeon, Korea). Bacterial strains were incubated in MRS broth (Difco) containing 0.05% (w/v) L-cysteine·HCl under anaerobic conditions overnight at 37 °C, and were subcultured in MRS broth and used for the incubation of ginseng extract or preparation of bacterial cell extract. *A. niger* KCTC 6906 was incubated in Potato Dextrose broth (Difco), and *A. usamii* var. *shirousamii* KCTC 6956 was incubated in Malt Extract broth (Difco) under aerobic conditions at 24 °C for 7 d with shaking. Each mycelia or culture broth was used for incubation of ginseng extract or preparation of a crude enzyme.

Preparation of Crude Microbial Enzymes Bacteria (1 l culture broth) were harvested by centrifugation (3000×*g* for 30 min at 4 °C), washed twice with 50 mM phosphate buffer (pH 6.0), resuspended in 50 ml 50 mM phosphate buffer and disrupted in a high pressure cell disrupter (Stansted Fluid Power, Essex, U.K.). The culture broth (500 ml) of *A. niger* KCTC 6906 was filtered, and then solid ammonium sulfate was added to the filtrate to give 80% saturation. The precipitate was collected by centrifugation (15000×*g* for 30 min at 4 °C), dissolved in 5 ml 50 mM phosphate buffer (pH 6.0), and dialyzed. The collected mycelia (500 ml culture broth) of *A. usamii* var. *shirousamii* KCTC 6956 was resuspended in 200 ml 50 mM phosphate buffer (pH 6.0) and disrupted in a cell disrupter (Bead-Beater, Biospec Products, Bartlesville, OK, U.S.A.) by glass beads (0.5 mm in diameter). The supernatant was then used as an enzyme solution after centrifugation (5000×*g* for 30 min at 4 °C).

Assay of β -Glucosidase Activity To determine the glycosidase activity, 80 μ l of cell-free extracts of the cultured microorganisms were added to 20 μ l 5 mM *p*-nitrophenyl- β -glucopyranoside, then the mixture was incubated at 37 °C for 5 min. The reaction was stopped by adding 100 μ l of 0.5 M Na₂CO₃. The released *p*-nitrophenol (pNP) was measured at 450 nm. One unit of enzyme activity was defined as the amount that released 1 μ mol pNP per minute, and specific activity was obtained per mg dry weight cell or mg protein.

Transformation of Ginsenosides by Crude Microbial Enzymes The reaction mixture (100–2050 μ l) containing 0.05 mg of each ginsenoside in 50 μ l 20 mM acetate buffer (pH 5.0) and 50–2000 μ l of crude microbial enzyme was in-

cubated at 37 °C for various time periods. The reaction mixture was extracted with 200 μ l of *n*-butanol, and the *n*-butanol fraction was analyzed by TLC and HPLC according to Bae *et al.* and Ko *et al.*^{12,14}

RESULTS

Transformation of Rb2 by Food Microbial Enzymes

Various transformed products of Rb2 produced by cell-free extracts of the experimental food microorganisms were confirmed by TLC and HPLC analyses, and their ginsenoside levels are shown in Table 2. The experimental ginsenosides were not transformed by boiled enzyme preparations (data not shown). When ginsenoside Rb2 reacted with enzymes from various food microorganisms, the intermediate and final hydrolysis products of Rb2 were dependent on the microorganisms used. For instance, 2 mU of *Bifidobacterium* sp. Int57 enzyme (Int57) transformed Rb2 into Rd or F2 by 24 h. When the incubation time was increased up to 48 h, Rd and F2 were transformed into compound K. From these results, the transformation pathways of Rb1 by Int57 was Rb2→Rd→F2→compound K as shown in Fig. 1. To reconfirm the above transformation process, we again tried having Rd or F2 react with Int57. The results matched the pathways shown in Fig. 1 (data not shown). The same strategy used for Int57 was applied to analyze the various transformation pathways of Rb2 by different experimental microorganisms (Fig. 1). *Bifidobacterium* sp. SJ32 enzyme (SJ32) also transformed Rb2 into compound K via Rd and F2, although Int57 was more active in the transformation of Rb2 into compound K than SJ32. *L. delbrueckii* enzyme (*L. delbrueckii*) transformed Rb2 into Rh2 via Rd and F2. *Bifidobacterium* sp. SH5 enzyme (SH5) transformed Rb2 into Rd, and then further transformed Rd into F2 without producing compound K. *A. niger* enzyme (*A. niger*) and *A. usamii* enzyme (*A. usamii*) transformed Rb2 into compound K via compound O and compound Y.

Transformation of Rc by Food Microorganisms Various transformed products and transformation pathways of Rc are shown in Table 3 and Fig. 2, respectively. Int57 and SJ32 transformed Rc into compound K via Rd and then F2, although Int57 was more active in the transformation of Rc into compound K than SJ32. SH5 also hydrolyzed the α -arabinofuranose of Rc to produce Rd, and then further transformed Rd into F2 without producing compound K. *L. del-*

Table 2. Concentrations of Ginsenosides Transformed from Rb2^{a)} by Cell-Free Extracts^{b)} from Various Food Microorganisms

Microbes	Transformed ginsenoside (μ M)													
	24 h							48 h						
	Rb2	Rd	CO ^{c)}	CY ^{d)}	F2	CK ^{e)}	Rh2	Rb2	Rd	CO	CY	F2	CK	Rh2
Int57	— ^{f)}	425	—	—	334	81	—	—	—	—	—	27	842	—
SJ32	314	89	—	—	427	—	—	—	—	—	—	79	794	—
SH5	75	788	—	—	17	—	—	—	84	—	—	803	—	—
<i>L. delbrueckii</i>	336	18	—	—	223	—	381	98	27	—	—	102	—	605
<i>A. niger</i>	—	—	515	343	—	—	—	—	—	—	784	—	88	—
<i>A. usamii</i>	—	—	514	329	—	—	—	—	—	—	763	—	95	—

a) Rb2: 910 μ M in the original sample. b) Specific activities of β -glucosidase of the prepared microbial cell-free extracts and amounts of enzymes used for the transformation reactions were: Int57, 0.50 mU/mg dry wt. cell, 2 mU; SJ32, 0.42 mU/mg dry wt. cell, 2 mU; SH5, 0.28 mU/mg dry wt. cell, 20 mU; *L. delbrueckii*, 0.15 mU/mg dry wt. cell, 20 mU; *A. niger*, 0.25 mU/mg protein, 10 mU; *A. usamii*, 0.23 mU/mg protein, 10 mU, respectively. c) CO: compound O. d) CY: compound Y. e) CK: compound K. f) Below 10 μ M. g) *L. delbrueckii*.

brueckii hydrolyzed the α -arabinofuranose of Rc to produce Rd, which was further transformed into Rh2 via F2. *A. niger* transformed Rc into compound K via compound Mc. *A. us- amii* ultimately transformed Rc into compound K, but the pathway was not determined in the present study.

DISCUSSION

Intestinal microflora can transform ginsenosides into more active forms after ingestion of ginsenosides.⁴⁾ Protopanaxadiol ginsenosides such as Rb1, Rb2, and Rc have previously been shown to be metabolized by human intestinal bacteria to their final derivative, compound K, with the degree of

transformation of the ginsenosides and production of compound K differing between *Eubacterium* sp., *Streptococcus* sp., and *Fusobacterium* K-60.^{3,4)} The efficiency of conversion and the transformation pathways may differ greatly due to the diversity of the resident microflora between individuals.⁵⁾ Therefore, a more uniform and targeted biological function may be achieved by providing specifically transformed ginsenosides. Few studies have been performed by edible microorganisms on the transformation of Rb2 and Rc, even though they belong to the major components of the ginsenosides.

Here we have focused on understanding the transformation

Fig. 1. Proposed Transformation Pathways of Rb2 by Cell-Extracts from Various Food Microorganisms

Fig. 2. Proposed Transformation Pathways of Rc by Cell-Extracts from Various Food Microorganisms

Table 3. Concentrations of Ginsenosides Transformed from Rc^{a)} by Cell-Free Extracts^{b)} from Various Food Microorganisms

Microbes	Transformed ginsenoside (μM)											
	24 h						48 h					
	Rc	Rd	F2	Mc	CK ^{c)}	Rh2	Rc	Rd	F2	Mc	CK	Rh2
Int57	— ^{d)}	554	214	—	91	—	—	—	172	—	701	—
SJ32	—	765	94	—	—	—	—	—	307	—	554	—
SH5	89	745	—	—	—	—	—	244	616	—	—	—
<i>L. del</i> ^{e)}	705	24	31	—	—	62	18	22	54	—	—	768
<i>A. niger</i>	15	—	—	832	—	—	—	—	—	735	88	—
<i>A. usamii</i>	471	269	14	31	27	—	20	33	—	324	473	—

a) Rc: 910 μM in the original sample. b) Specific activities of β -glucosidase of the prepared microbial cell-free extracts were the same as in Table 2. Amounts of enzymes used for the transformation reactions were: Int57, 2 mU; SJ32, 2 mU; SH5, 20 mU; *L. delbrueckii*, 20 mU; *A. niger*, 20 mU; *A. usamii*, 20 mU, respectively. c) CK: compound K. d) Below 10 μM . e) *L. del*: *L. delbrueckii*.

of Rb2 and Rc by edible food microorganisms. The only difference between Rb2 and Rc is the configuration of the arabinose unit attached at C-20 of the ginsenoside linked by glucose. In Rb2 the arabinose is a pyranose form, and in Rc it is a furanose form. In this study, the transformation pathways of Rb2 and Rc were similar in most of the microorganisms tested. The strains Int57, SJ32, and SH5 cleaved the arabinose unit of α -L-arabinose(1—6)- β -D-glucose-O- at C-20 of Rb2 and Rc better than the glucose unit of β -D-glucose(1—2)- β -D-glucose-O- at C-3. In contrast, *A. niger* cleaved β -D-glucose(1—2)- β -D-glucose-O- at C-3 better than the arabinose unit of Rb2. It was of interest that *L. delbreuckii* produced Rh2 from both Rb2 and Rc, unlike the other experimental microorganisms.

This is the first report of the production of Rh2 from Rb2 by bacteria. Previously, the biotransformation of Rb1 into Rh2 via Rd and Rg3 by the fungus *Rhizopus stolonifer* was reported.¹⁵⁾ Bae *et al.* reported that Rb1 and Rb2 were not metabolized to Rh2 by human intestinal bacteria.¹⁴⁾ They suggested that Rb1 and Rb2 should be transformed into Rg3 in the stomach to be absorbed in the form of Rh2, based on their observation that human intestinal bacteria such as *Bacteroides* sp., *Fusobacterium* sp., and *Bifidobacterium* sp. were able to transform Rg3 into Rh2. Otherwise, Rb1 and Rb2 were presumed to be metabolized to compound K in the human intestine. Since *L. delbreuckii* is a safe bacteria, *L. delbreuckii*-treated ginsenosides would be an effective source for providing Rh2 when the intake of Rh2 is desired.

The functions of Rh2 and compound K, which are the specific final products for the respective enzymes in this study, have been reported. The major activities for Rh2 include inhibition of the initiation and progression of tumor cells.¹⁶⁾ Rh2 inhibits the growth of B16 melanoma cells. The production of nitric oxide by IFN gamma plus LPS-treated macrophages is markedly reduced by Rh2, but is not inhibited by Rb1, Rc, or Re.¹⁷⁾ Compound K is known to induce an antitumorigenic or anticarcinogenic effect by blocking tumor invasion or preventing chromosomal activation and tumorigenesis.¹⁸⁾ From the above reports, the transformed products of Rb2 and Rc by the food microbial enzymes used in this experiment would be used as an anticarcinogenic food compound. Our experimental demonstration that various edible microorganisms produce specific forms of ginsenosides

may indicate that it is feasible to develop a specific bioconversion process to obtain specifically designed functional products by the appropriate combination of ginsenoside substrates and specific microbial enzymes.

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