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Microbial Conversion of Major Ginsenoside Rb_1 to Pharmaceutically Active Minor Ginsenoside Rd

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More than seventy strains of aerobic bacteria showing β-glucosidase activity were isolated from a ginseng field, using a newly designed Esculin-R2A agar, and identified by their 16S rRNA gene sequences. Of these microorganisms, twelve strains could convert the major ginsenoside, Rb_1, to the pharmaceutically active minor ginsenoside Rd. Three strains, *Burkholderia pyrrocinia* GP16, *Bacillus megaterium* GP27 and *Sphingomonas echinoides* GP50, were phylogenetically studied, and observed to be most potent at converting ginsenoside Rb_1 almost completely within 48 h, as shown by TLC and HPLC analyses.

Key words: conversion, ginseng, ginsenoside, Rb_1, Rd

Ginseng is one of the most popular medicinal plants, the roots of which have long been traditionally used for strengthening immunity, providing nutrition and recovering health from fatigue. Recently, pharmaceutical activities of ginseng roots have been proven by many researches, and ginseng has become a world-famous medicinal plant. Ginseng roots contain various pharmaceutical components-ginsenosides (saponins), polyacetylenes, polyphenolic compounds and acidic polysaccharides, and among the components, ginsenosides are the most pharmaceutically active. Until now, 38 ginsenosides have been isolated from ginseng roots, with five major ginsenosides (ginsenosides Rb_1, Rb_2, Rc, Re and Rg_1) constituting more than 80% of the total ginsenosides (Kim *et al.*, 1987).

In the recent decades, many studies have focused on the pharmaceutical activities of the minor ginsenosides, such as ginsenosides Rd, Rg_3, Rh_2 and ginsenoside K (compound K), as their activities are found to be superior to those of the major ginsenosides. These minor ginsenosides are present in ginseng only in small percentages and known to be produced by hydrolysis of the sugar moieties of the major ginsenosides. Therefore, many studies have aimed to convert major ginsenosides to the more active minor ginsenosides, with methods, such as heating, acid treatment and enzymatic conversion, having been developed. Heating and acid treatment also degrade other active minor ginsenosides and acidic polysaccharides by randomly hydrolyzing glycosidic bonds, which can remove the other pharmaceutical activities of ginseng. However, the enzymatic conversion for the appropriate sugar-hydrolysis of a specific position is desirable for the production of active minor ginsenosides. Many studies for the production of the active minor ginsenosides Rg_3, Rh_2 and ginsenoside K have been performed using microbial enzymes.

The minor ginsenoside, Rd, can be produced by hydrolyzing and removing a sugar moiety from the major ginsenosides, Rb_1, Rb_2 and Rc. Ginsenosides Rb_1 is especially the most abundant (23%) (Kim *et al.*, 1987) of all the ginsenosides, and its structure can be easily converted to ginsenoside Rd by hydrolysis of one glucose moiety (Fig. 1).

The minor ginsenoside, Rd, has been known to enhance the differentiation of neural stem cells, while other ginsenosides induce no differentiation of neurons (Shi *et al.*, 2005) and are known to protect neural systems against neurotoxicity by attenuating NO overproduction (Choi *et al.*, 2003). The pharmaceutical property of ginseng roots in protecting neurons from neurotoxic chemicals, such as kainic acid, is attributed mostly to ginsenoside Rd (Lee *et al.*, 2003).

Ginsenoside Rd has been known to decrease levels of urea nitrogen and creatinine in the kidney, and has demonstrated protection against the renal dysfunction caused by ischemia (= local anemia) and recirculation (Yokozawa *et al.*, 1998), and is also known to protect the kidney from apoptosis and DNA fragmentation caused by chemical drugs and cancer drugs (Yokozawa and Owada, 1999; Yokozawa and Liu, 2000; Yokozawa and Dong, 2001).

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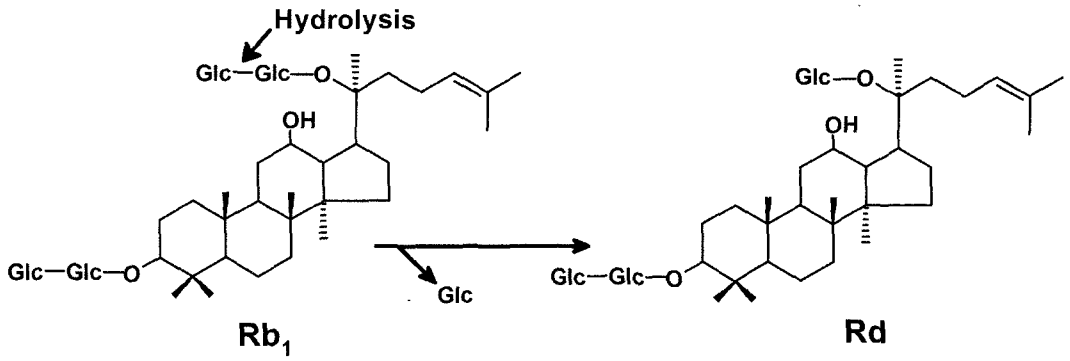

Fig. 1. Microbial conversion (hydrolysis) of ginsenoside Rb_1 to ginsenoside Rd.

Ginsenoside-Rd has been known to arrest the aging process of the suppressing antioxidative defence system and lipid peroxidation by elevating the GSH/GSSG ratio of glutathione and increasing the activities of glutathione peroxidase and glutathione reductase, which are both significantly lower in old organisms (Yokozawa *et al.*, 2004). Ginsenoside Rd is also known to help learning and memory functions in mice (Chen *et al.*, 2001) and prevent contraction of blood vessel; therefore, preventing blocking of blood circulation (Zeng *et al.*, 2003).

In the present study, major ginsenoside Rb_1 was converted to minor ginsenoside Rd by the action of microbial β-glucosidase. First, the β-glucosidase-producing bacteria were isolated from a ginseng-growing field. Second, all the β-glucosidase-producing bacteria were tested to verify their ginsenoside Rb_1-converting activity using TLC and HPLC.

Materials and Methods

β-Glucosidase activity test

β-glucosidase (sigma, USA) was purchased commercially, and its ginsenoside Rb_1-converting activity tested. One unit of β-glucosidase and 1mM of ginsenoside Rb_1 were dissolved in 1.5 ml of phosphate buffer (50 mM, pH 7.0). The reaction mixture was incubated for 24 h at 30°C and analyzed by TLC, as below.

Screening of microorganisms producing β-glucosidase

Esculin-R2A Agar was used to isolate β-glucosidase-producing microorganisms. Esculin-R2A Agar contains (per 1 L): esculin 1 g and ferric citrate 0.5 g with R2A agar (Difco, USA). The microorganisms producing β-glucosidase and that hydrolyze esculin appeared as colonies surrounded by a reddish-brown to dark brown zone. Microorganisms were isolated from the soil of a ginseng field via direct plating onto Esculin-R2A Agar. Single colonies from these plates were purified by transferring onto new plates, which were then subjected to an additional incubation for 3 days at 30°C.

Identification of isolated bacteria

Genomic DNAs of the β-glucosidase-producing bacteria were extracted and purified using a Genomic DNA Isolation Kit (Core Bio System, Korea). The 16S rRNA genes were amplified using the universal bacterial primer set, 9F and 1512R (Weisburg *et al.*, 1991), and the purified PCR products directly sequenced by Genotec, Korea (Kim *et al.*, 2005). The 16S rRNA gene sequences of the β-glucosidase-producing bacteria were blasted in the NCBI database, and the closest type strains determined.

Assay of ginsenoside Rb_1-converting activity of the β-glucosidase-producing bacteria

The reaction mixture, containing 200 μl of 1 mM ginsenoside Rb_1 and 200 μl of bacterial suspension cultured in Nutrient broth, was incubated for 48 h at 30°C, then extracted twice with 200 μl of butanol, evaporated and redissolved in methanol.

TLC analysis

TLC was performed on silica gel 60 F_{254} plates. A mixture of $CHCl_3$-MeOH-H_2O (65:35:10 v/v/v, lower phase) was used as the developing solvent. The spots on the TLC plates were detected by spraying 10% H_2SO_4, followed by heating at 110°C for 5 min.

HPLC analysis

The reaction mixture prepared for TLC analysis was separated by HPLC, using an Alltech Prevail Carbohydrate ES column (4.6 × 250 mm), using gradient conditions with acetonitrile-water-isopropanol (80:5:15, v/v/v) (solvent A) and acetonitrile-water-isopropanol (80:20:15, v/v/v) (solvent B). The ratios of solvent A / solvent B were 70/30, 0/100, 0/100 and 70/30, at running times of 0, 20, 50 and 60 min, respectively, at a flow rate of 0.8 ml/min, and with ELSD detection.

Phylogenetic study

The full sequences of the 16S rRNA genes were compiled with the SeqMan software and edited using the BioEdit program (Hall, 1999). The 16S rRNA gene sequences of

the related taxa were obtained from GenBank. Multiple alignments were performed with the CLUSTAL X program (Thompson *et al.*, 1997). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). The phylogenetic tree was constructed via the neighbor-joining method (Saitou and Nei, 1987)

Table 1. β-glucosidase producing microorganisms. 77 strains were screened using Esculin-R2A agar, with twelve strains found that converted ginsenoside Rb_1 to ginsenoside Rd

Class	Names	Strains	Rb_1 → Rd Hydrolysis
	Arthrobacter methylotrophus	GP34	-
	Arthrobacter nitroguajacolicus	GP23	-
	Cellulomonas flavigena	GP42	-
	Microbacterium phyllosphaerae	GP63	-
	Microbacterium resistens	GP05	-
	Streptomyces bikiniensis	GP35	-
Actinobacteria	*Streptomyces galbus*	GP08	-
	Streptomyces galilaeus	GP38	-
	Streptomyces lincolnensis	GP12, GP19	-
	Streptomyces nodosus	GP25	-
	Streptomyces olivochromogenes	GP39, GP62	-
	Streptomyces tauricus	GP30, GP36, GP70	-
	Terrabacter tumescens	GP22, GP28	-
	Bacillus megaterium	GP27	+
	Bacillus megaterium	GP11, GP74, GP78	-
	Bacillus simplex	GP17, GP18	-
	Bacillus subtilis	GP06, GP75	-
	Paenibacillus amylolyticus	GP31, GP40, GP41	+
	Paenibacillus amylolyticus	GP58	-
Bacilli	*Paenibacillus glycanilyticus*	GP02	+
	Paenibacillus kobensis	GP13, GP14, GP15, GP24	-
	Paenibacillus macerans	GP29	-
	Paenibacillus naphthalenovorans	GP20, GP21	-
	Paenibacillus pabuli	GP32	-
	Paenibacillus validus	GP64, GP67	-
	Planococcus antarcticus	GP65	-
	Caulobacter leidyia	GP45	-
	Sphingomonas echinoides	GP46, GP47, GP50, GP53	+
Proteobacteria (alpha)	*Sphingomonas echinoides*	GP48, GP49, GP52, GP54, GP79	-
	Sphingomonas stygialis	GP44, GP51	-
	Sphingomonas yabuuchiae	GP43	-
Proteobacteria (beta)	*Burkholderia pyrrocinia*	GP16	+
	Buttiauxella izardii	GP07, GP26	-
	Enterobacter asburiae	GP37, GP55, GP56, GP69, GP71, GP72	-
	Escherichia fergusonii	GP77	-
	Escherichia senegalensis	GP87	+
	Escherichia vulneris	GP59, GP60	-
Proteobacteria (gamma)	*Frateuria aurantia*	GP33	+
	Klebsiella pneumoniae	GP81, GP83	-
	Kluyvera cochleae	GP66	-
	Pseudomonas nitroreducens	GP76	-
	Raoultella planticola	GP80, GP82	-
	Serratia fonticola	GP01	-
Bacteroidetes/Chlorobi	*Cytophaga arvensicola*	GP84	-

employing the MEGA 2 Program (Kumar *et al.*, 2001). A bootstrap analysis with 1,000 replicates was also conducted to obtain confidence levels for the branches (Felsenstein, 1985). The closest type strains were included in the phylogenetic trees.

Results

Initial screening of β-glucosidase-producing microorganisms

Seventy-seven microorganisms were initially screened, using the Esculin-R2A agar, for their ability to produce β-glucosidase. The black colonies on the Esculin-R2A agar that showed β-glucosidase activity were picked and transferred to the fresh Esculin-R2A agar. Pure cultures were checked for shape, color and size of colonies.

Identification of isolated strains

All the isolated strains were identified using their 16S rRNA gene sequences. 16S rRNA partial sequences, with sizes around 700 bp, were blasted in the NCBI database, with the closest type strains discovered (Table 1). Most strains are classified as Gram-positive *Actinobacteria*, *Bacilli* and *Proteobacteria*. More precisely, the isolated β-glucosidase-producing bacteria belonged to *Streptomyces*, *Bacillus*, *Paenibacillus*, *Sphingomonas*, *Enterobacter* and *Escherichia* genera. The sequence similarity of their 16S rRNA genes to those of their closest type strains ranged from 96.0 to 100.0%.

TLC assay of the conversion of ginsenoside Rb_1

All the β-glucosidase-producing strains were assayed to verify their hydrolytic activity for converting ginsenoside Rb_1. Ginsenoside Rb_1 was metabolized in culture broths containing the screened microorganisms, with metabolites produced due to microbial activity analyzed by TLC. Of the 77 β-glucosidase-producing strains, twelve were shown to be able to convert ginsenoside Rb_1 to ginsenoside Rd. Their TLC results are shown in Fig. 2. They converted ginsenoside Rb_1 to ginsenoside Rd, but some strains produced less polar metabolites, which are spotted in the upper part of the TLC. Of the ginsenoside-converting strains, *Burkholderia pyrrocinia* GP16, *Bacillus megaterium* GP27 and *Sphingomonas echinoides* GP50 were found to be the most potent strains for almost completely converting ginsenoside Rb_1 to ginsenoside Rd. Strain GP50 produced a less polar metabolite, with an Rf value similar to that of ginsenoside K (compound K).

HPLC assay of the conversion of ginsenoside Rb_1

An HPLC assay was performed to verify the conversion of ginsenoside Rb_1. All the strains with positive results in TLC were subjected to HPLC. As shown in Fig. 3, new peaks, which were considered as minor ginsenosides, were observed in HPLC patterns **C** and **D**. These new peaks were not formed in the reaction mixture with the negative strains in the TLC analysis results (**B**). Two new peaks were observed in HPLC pattern C from the *Sphingomonas echinoides* GP50 broth. One of the new peak had a retention time consistent with that of ginsenoside-Rd (a main product), but the other new peak showed a much shorter retention time, similar to that of ginsenoside K (compound K). Complete disappearance of the ginsenoside Rb_1 was accompanied by the marked accumulation of ginsenoside Rd in the incubated reaction mixture. With HPLC pattern **D**, a peak indicating a significant amount of ginsenoside Rb_1 was detected. It was observed that the conversion reaction of other less reactive strains resulted in a portion of the ginsenoside Rb_1 remaining unconverted.

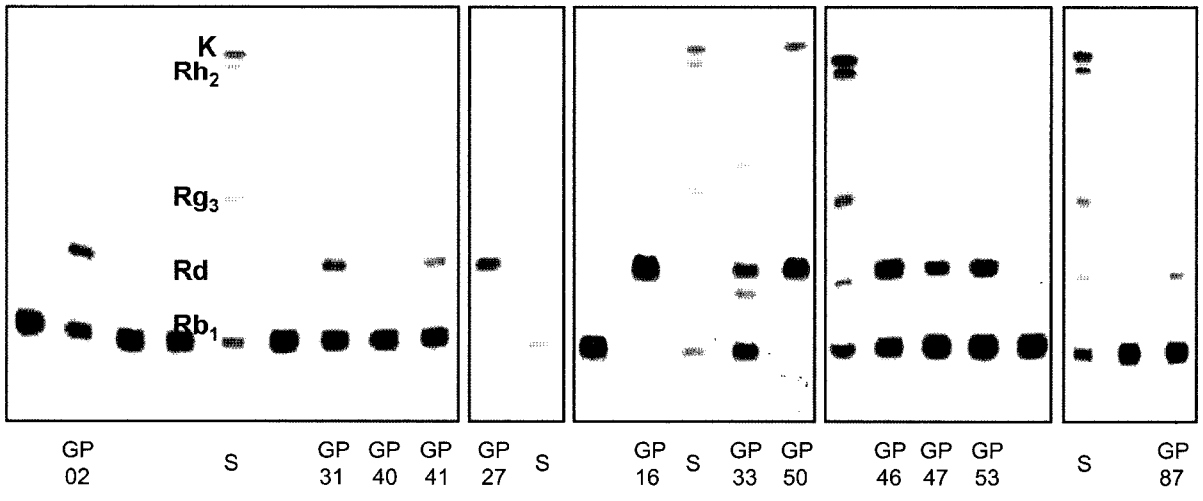

Fig. 2. TLC chromatograms of the ginsenoside Rb_1 metabolites formed by β-glucosidase producing strains. Twelve ginsenoside Rd-forming strains, with other strains with no activity (negative control) are shown. Only the names of the twelve strains forming ginsenoside Rd are shown in the TLC chromatograms below. Those of the negative control are not shown. s: standard ginsenosides (upwards, five ginsenoside Rb_1, Rd, Rg_3, Rh_2 and K (compound K)).

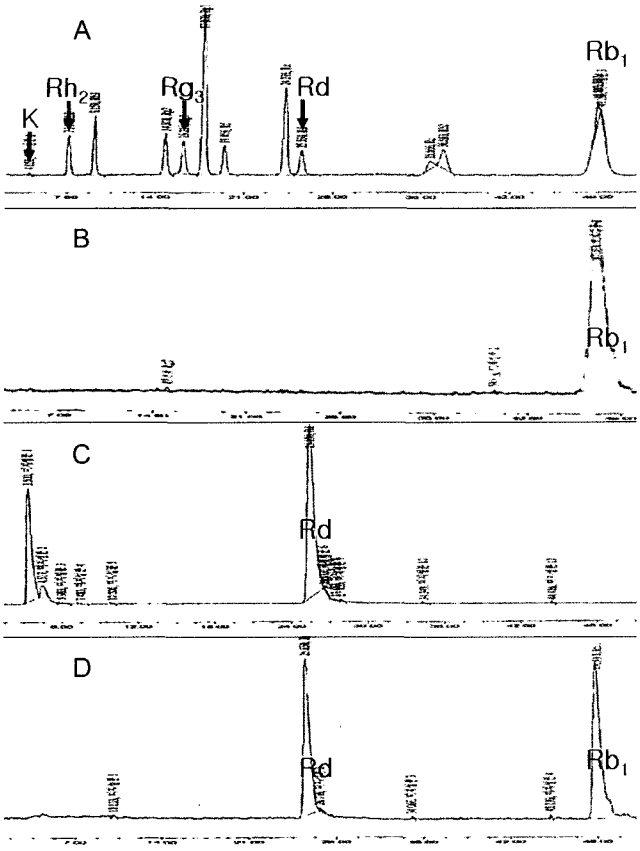

Fig. 3. HPLC chromatograms of ginsenoside Rd formed by β-glucosidase-producing strains. **A**, ginsenoside standards; **B**, ginsenoside Rb_1 remaining (negative control); **C**, metabolites formed by the potent ginsenoside Rb_1-converter GP50; **D**, metabolites formed by the less reactive ginsenoside Rb_1-converter GP46. The conversion yields of ginsenoside Rb_1 to ginsenoside Rd were 65 and 48% by strains GP50 and GP46, respectively.

Phylogeny of the three strains to potently convert ginsenoside Rb_1

The three strains that potently converted ginsenoside Rb_1 to ginsenoside Rd were studied phylogenetically. The 16S rRNA gene sequences of the three strains, GP16, GP27 and GP50, were aligned together with those of the type strains found to have the closest taxonomic positions. The 16S rRNA gene sequences of the related taxa were obtained from GenBank. Strain GP16 was determined as belonging to the beta subclass of *Proteobacteria,* with the highest degree of 16S rRNA gene sequence similarity found with *Burkholderia pyrrocinia* LMG 14191^T, at 99.8% (Fig. 4). Strain GP27 was determined as belonging to *Bacilli,* with the highest 16S rRNA gene sequence similarity with *Bacillus megaterium* IAM 13418^T, at 99.7%. Strain GP50 was determined as belonging to alpha-4 subclass of *Proteobacteria*, with the highest 16S rRNA gene sequence similarity with *Sphingomonas echinoides* ATCC 14820^T, at 99.5%. In view of the taxonomic evaluation, the strains showing less than 1% differences in their 16S rRNA gene sequence similarities with the corresponding type strains were assumed to be the same species as the type strains. In the phylogenetic trees (Fig. 4), all three strains clearly belonged to the same lineage as their corresponding type strains, as evidenced by the high bootstrap values.

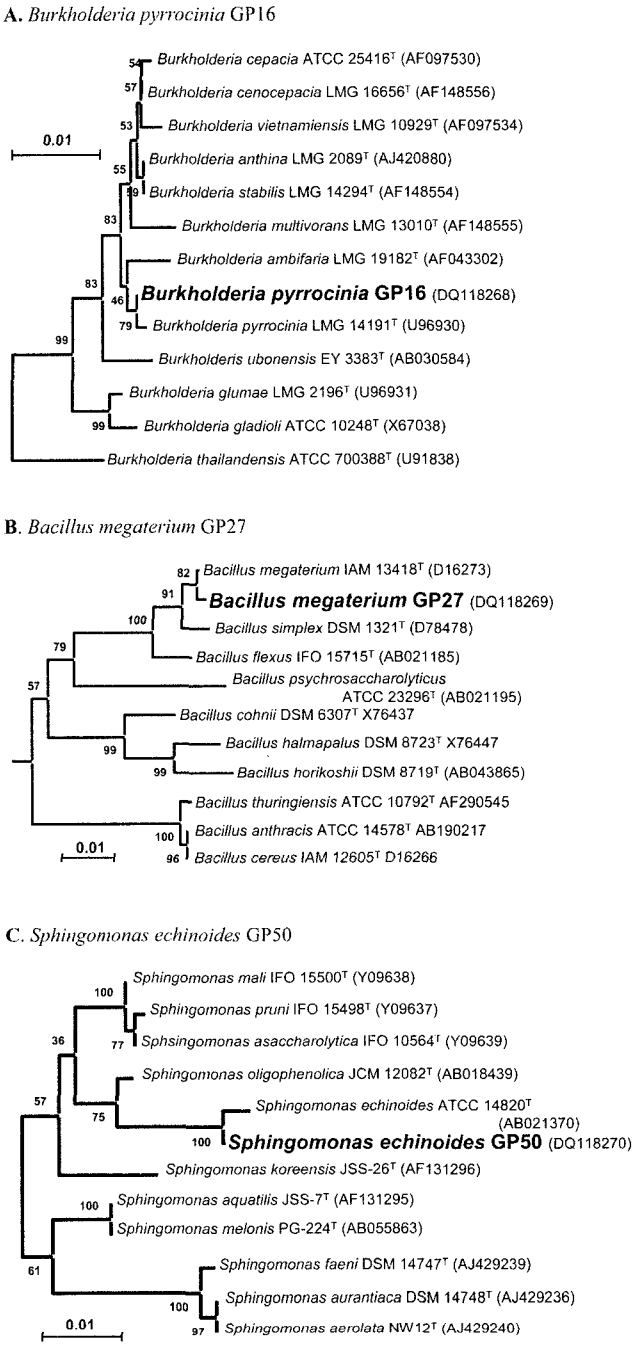

Fig. 4. Phylogenetic trees based on the 16S rRNA gene sequences, showing the phylogenetic relationships of the potent Rb_1-converting strains, GP16, GP27 and GP50, with their closest type strains (neighbor-joining method). A bar represents 0.01 substitutions per nucleotide position. **A.** *Burkholderia pyrrocinia* GP16, **B.** *Bacillus megaterium* GP27, **C.** *Sphingomonas echinoides* GP50.

Discussion

Ginsenoside Rb_1 can be converted to the minor ginsenoside Rd by simple hydrolysis and loss of a glucose moiety from the C-20 position of ginsenoside aglycone. Of the five major ginsenosides, only three, ginsenosides Rb_1, Rb_2 and Rc, are of the protopanaxadiol (PPD)-type and can be converted to ginsenoside Rd. Because ginsenoside Re and Rg_1 are of the protopanaxatriol (PPT)-type, their hydrolysis products cannot be PPD-type ginsenoside Rd. The aglycone of ginsenosides Rb_1, Rb_2 and Rc is the same as that of ginsenoside Rd, with these compounds differing only in the presence of 20-*O*-α-L-glucopyranoside, arabinopyranoside and arabinofuranoside, respectively. Many enzymes hydrolyzing the above glycosidic bonds are known, with β-glucosidase regarded as being the most useful (Bae *et al.*, 2000; Park *et al.*, 2001). In the present study, attempts were made to screen many active β-glucosidase-producing bacteria.

The Esculin-R2A agar was specially designed to screen β-glucosidase-producing bacteria. Esculin was needed to detect the β-glucosidase activity, with R2A agar being optimal for the cultivation of the bacteria dominant in soil, as this agar had a low concentration of nutrients.

This study was the first trial to obtain aerobic bacteria from soil that were capable of converting ginsenoside Rb_1. There have been previous reports on microbial sources able to convert the major ginsenoside Rb_1 to ginsenoside Rd. β-Glucosidases from the human intestinal bacteria, *Bifidobacterium* sp., *Eubacterium* sp. and *Fusobacterium* sp., hydrolyzed ginsenoside Rb_1 to ginsenoside Rd (Bae *et al.*, 2000; Park *et al.*, 2001). Fungal conversion, using fungi *Rhizopus stolonifer* and *Curvularia lunata*, has been also reported (Dong *et al.*, 2003). Because aerobic bacteria grow faster and produce enzymes in greater quantities than human intestinal bacteria (Coskun and Öndül, 2004; Yoon *et al.*, 2004) and fungi, aerobic bacteria can be more effectively used for large scale enzyme preparation. Furthermore, the cultivation of human intestinal bacteria requires anaerobic space and a medium with a high concentration of nutrients, and their cultivation is not as simple as that of aerobic bacteria.

The ginsenoside Rb_1-converting enzymes of the three potent strains, GP16, GP27 and GP50, can be applied to industrial use for the production of ginsenoside Rd after the key enzymes for the conversion have been purified and characterized, which should be the subject of further study. The substrate specificity of the enzymes for other major ginsenosides Rb_2 and Rc must also be determined. In further studies, a kinetic analysis of the conversion over a longer period will be required. For example, strain GP50 produced ginsenoside Rd as a main product, with ginsenoside K (compound K) as a minor product, after incubation for 48 h. It is assumed the strain GP50 would be able to produce a higher amount of ginsenoside K with a longer incubation time, as ginsenoside K can be produced by further hydrolysis of the sugar from ginsenoside Rd.

At present, no evidence exists that the β-glucosidases produced from the strains play a significant role in the conversion of ginsenoside Rb_1, as other enzymes secreted from the β-glucosidase-producing microorganisms can also hydrolyze ginsenoside Rb_1. Purification of the ginsenoside Rb_1-converting enzyme would verify the enzymatic reaction. In fact, most research on the conversion of ginsenoside has been performed with purified enzymes. In China, ginsenoside-α-arabinofuranase hydrolyzing ginsenoside Rc to Rd has been purified from fresh ginseng root (Zhang *et al.*, 2002), and gypenoside-α-rhamnosidase hydrolyzing gypenoside-5 to ginsenoside Rd has also been purified and characterized (Yu *et al.*, 2004). However, until now, only one enzyme able to convert ginsenoside Rb_1 to ginsenoside Rd had been purified from human intestinal bacteria (Park *et al.*, 2001). Furthermore, the above mentioned ginsenoside-α-arabinofuranase is the only enzyme produced by ginseng itself. Therefore, microorganisms are the only sources for the enzymes to convert ginsenoside Rb_1 to ginsenoside Rd.

Besides ginsenoside Rd, some of the screened bacteria were able to convert ginsenoside Rb_1 to other minor ginsenosides, such as Rg_3, Rh_2 and ginsenoside K (compound K). Further studies with these ginsenoside Rb_1-converging bacteria are currently being undertaken.

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