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Characterization of Recombinant β-Glucosidase from *Arthrobacter chlorophenolicus* and Biotransformation of Ginsenosides Rb_1, Rb_2, Rc, and Rd

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The focus of this study was the cloning, expression, and characterization of recombinant ginsenoside hydrolyzing *β*-glucosidase from *Arthrobacter chlorophenolicus* with an ultimate objective to more efficiently bio-transform ginsenosides. The gene *bglAch*, consisting of 1,260 bp (419 amino acid residues) was cloned and the recombinant enzyme, over-expressed in *Escherichia coli* BL21 (DE3), was characterized. The GST-fused BglAch was purified using GST·Bind agarose resin and characterized. Under optimal conditions (pH 6.0 and 37°C) BglAch hydrolyzed the outer glucose and arabinopyranose moieties of ginsenosides Rb_1 and Rb_2 at the C20 position of the aglycone into ginsenoside Rd. This was followed by hydrolysis into F_2 of the outer glucose moiety of ginsenoside Rd at the C3 position of the aglycone. Additionally, BglAch more slowly transformed Rc to F_2 via C-Mc_1 (compared to hydrolysis of Rb_1 or Rb_2). These results indicate that the recombinant BglAch could be useful for the production of ginsenoside F_2 for use in the pharmaceutical and cosmetic industries.

Keywords: biotransformation, *β*-glucosidase, recombinant enzyme, minor ginsenoside, *Arthrobacter chlorophenolicus*

Introduction

Ginseng (*Panax ginseng* C.A. Meyer) is an important herbal medicine that has been widely used for thousands of years in East Asia and has been popularized in the West during recent decades (Cho, 2012; Park *et al*., 2012). Ginsenosides are the major active components of ginseng, and they appear to be responsible for the principle pharmacological activities of ginseng, including anti-fatigue, anti-neoplastic, anti-diabetic, anti-inflammation, and anti-oxidant effects (Leung and Wong, 2010; Lee *et al*., 2011; Yuan *et al*., 2011). After oral intake of ginseng, the major ginsenosides are hydrolyzed through human intestinal digestion into the more active minor ginsenosides, which are easily absorbed. For instance, ginsenoside Rb_1 is converted to ginsenosides Rd, F_2, compound K, and aglycone by intestinal micro-flora (Akao *et al*., 1998; Tawab *et al*., 2003; Choi *et al*., 2011). Therefore, converting the major ginsenosides, which account for more than 80% of the total ginsenosides (Attle *et al*., 1999; Christensen, 2009), to highly active minor ginsenosides such as F_2, Rg_3, Rh_2, C-K, Rh_1, and F_1 is highly significant for the pharmaceutical industry (Park *et al*., 2008; Choi *et al*., 2009; Leung and Wong, 2010; Lee *et al*., 2011; Mai *et al*., 2012; Shin *et al*., 2012).

The minor ginsenosides can be produced by hydrolyzing sugar moieties from the major ginsenosides such as Rb_1, Rb_2, Rc, Rd, Re, Rg_1 (Park *et al*., 2010). To date, several methods to produce pure ginsenosides have been developed (e.g., heating, acid treatment, and enzymatic transformation). The enzymatic methods are considered the most promising as they have the advantages of fewer byproducts, superior environmental protection, and better stereo-specificity (An *et al*., 2010; Wang *et al*., 2011; Kim *et al*., 2012; Cui *et al*., 2013a). In particular, the recombinant enzymes exhibit higher selectivity and efficiency than those isolated and purified from cultivated microorganisms (Hong *et al*., 2012; Cui *et al*., 2013b). Large-scale (100 g) production of particular ginsenosides is now available using enzymatic transformation methods (Kim *et al*., 2013).

In this study, we report the cloning of a new gene encoding ginsenoside-hydrolyzing β-glucosidase (BglAch) from *Arthrobacter chlorophenolicus*, which was isolated from soil (Westerberg *et al*., 2000). It possesses 72 kinds of glycoside hydrolases within 31 glycoside hydrolase families in its genomic DNA [Carbohydrate-Active enZymes (CAZY) database (http://www.cazy.org)]. The enzymatic properties and substrate specificities of a recombinant enzyme belonging to glycoside hydrolase family 1 was thoroughly investigated.

Materials and Methods

Chemicals

Ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rg_1, Rg_3(*S*), F_2, Rh_2(*S*),

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and C-K were purchased from Nanjing Co. Ltd. and gypenoside XVII, compound O (C-O), compound Mc_1 (C-Mc_1) were obtained by our group as described by Wang *et al.* (2011). All the chemicals used in this study were at least analytical reagent grade, and the sources are noted individually in the Methods section.

Bacterial strains, vectors and media

Arthrobacter chlorophenolicus KACC 20538^T, used as a source for cloning of the *β*-glucosidase gene, was cultivated on R2A agar (BD, USA) under aerobic conditions at 30°C. *Escherichia coli* BL21 (DE3) and the pGEX 4T-1 plasmid (GE Healthcare, USA) for gene cloning and expression was cultivated in a Luria-Bertani (LB) medium with ampicillin (100 mg/L).

Phylogenetic analysis of BglAch

A database homology search was performed with the BLAST program provided by NCBI. Sequences of the characterized glycosyl hydrolases were obtained from the CAZY database, and multiple alignments were performed using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999), and a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with a poisson model and pairwise deletion in the MEGA5 Program (Tamura, 2011), and with bootstrap values based on 1000 replicates (Felsenstein, 1985).

Molecular cloning, expression and purification of recombinant BglAch

Genomic DNA of *Arthrobacter chlorophenolicus* KACC 20538^T was extracted by using a genomic DNA extraction kit (Solgent, Korea). The gene, termed *bglAch*, encoding β-glucosidase was amplified from the genomic DNA as a template via a polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Solgent). The sequences of the oligonucleotide primers used for the gene cloning were based on the DNA sequence of *β*-glucosidase (GenBank accession number: ACL 38420). Forward (5′-G GTT CCG CGT GGA TCC ACC AAC CAG TTT CCG CAG GAC TTC-3′) and reverse (5′-G ATG CGG CCG CTC GAG TCA GGC GGG TTG GGC CAC CTG GGA-3′) primers were designed as primers to introduce the *Bam*HI and *Xho*I restriction sites (underlined), respectively, and were synthesized by Bioneer Co. Ltd. (Korea). The amplified DNA fragment obtained from the PCR was purified and inserted into the pGEX 4T-1 GST Glutathione S-transferase (GST) fusion vector digested with *Bam*HI and *Xho*I using an EzCloning Kit (Enzynomics Co. Ltd., Korea). The resulting recombinant pGEX-*bglAch* was transformed into *E. coli* BL21(DE3). The *E. coli* BL21(DE3) harboring the recombinant plasmid was grown in an LB-ampicillin medium at 37°C until the culture reached an OD_{600} of 0.6, at which point protein expression was induced through the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The bacterial cells were incubated for a further 18 h at 25°C and were then harvested by centrifugation at 12,000 rpm for 10 min at 4°C. The cells were washed twice with 50 mM sodium phosphate buffer (pH 7.0, 5 mM EDTA, and 1% Triton X-100) and then resuspended in 50 mM sodium phosphate buffer (pH 7.0). The cells were disrupted by ultrasonication (Vibra-cell; Sonics & Materials, USA) and the intact cells and debris were removed by centrifugation at 12,000 rpm for 10 min at 4°C. The GST-tagged fusion protein was purified by GST·Bind agarose resin (Elpis Co. Ltd., Korea). The GST·Tag sequence was removed from the GST·Bind agarose resin after being incubated with thrombin. The purified recombinant protein used for the assay has an extra two residues (Arg-Ser) at the N-terminus of wild type BglAch. The final buffer is 50 mM sodium phosphate (pH 7.0). The homogeneity of the protein was assessed by 10% SDS-PAGE and EZ-Gel staining solution (Daeillab Co. Ltd., Korea). Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Pierce, USA), with bovine serum albumin (Sigma) as the standard. All assays were performed in triplicate.

Characterization of recombinant BglAch

The specific activity of purified BglAch was determined by using *p*-nitrophenyl-*β*-D-glucopyranoside (PNPG) as substrate in 50 mM sodium phosphate buffer (pH 6.0) at 37°C. The release of *p*-nitrophenol was immediately measured using a microplate reader at 405 nm (Bio-Rad Model 680, USA). One unit of activity was defined as the amount of protein required to generate 1 μmol of *p*-nitrophenol per minute. To check the optimum condition for the specific enzyme activity, pH, temperature, metals and chemical reagents were investigated as previously described by An *et al.* (2010). The effect of pH on enzymatic activity was determined in the following buffers (each at 50 mM): KCl-HCl (pH 2.0), glycine-HCl (pH 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0), Tris-HCl (pH 8.0, and 9.0) and glycine-sodium hydroxide (pH 10.0). The results are expressed as a percentage of the activity obtained at the optimum pH. Substrate preference was examined by using chromogenic *o*-nitrophenyl (ONP) and *p*-nitrophenyl (PNP).

A kinetic study was performed with freshly purified enzymes using PNP-β-D-glucopyranoside (pNPGlc) at concentrations from 1.0 mM to 20 mM. One unit of activity was defined as the amount of protein required to generate 1 μmol of *p*-nitrophenol per minute. All assays were performed in triplicate.

Enzymatic hydrolysis of ginsenosides

To investigate the biotransformation ability of recombinant *β*-glucosidase, BglAch, 6 different ginsenosides [Rb_1, Rb_2, Rc, Rd, Re, and Rg_1] were evaluated as substrates. The initial biotransformation experiments using ginsenoside Rb_1 as the substrate revealed that having GST fused to the BglAch did not affect the activities of BglAch. The crude enzyme solutions at concentrations of 0.1 mg/ml in 50 mM sodium phosphate buffer (pH 6.0) were reacted with an equal volume of Rb_1, Rb_2, Rc, and Rd solution at a concentration of 0.1% (wt/vol) in 50 mM of sodium phosphate buffer (pH 6.0) at 37°C. Samples were withdrawn at regular intervals. An equal volume of water-saturated *n*-butanol was added to stop the reaction, and the reactant present in the *n*-butanol fraction

was analyzed by TLC after pretreatment. For the HPLC analyses, an equal volume of methanol was added to stop the reaction. After centrifugation at 15,000×g for 10 min, the supernatant was used for the sample analyses.

Analysis of ginsenosides by thin-layer chromatography (TLC)

TLC was performed using $60F_{254}$ silica gel plates (Merck, Germany) with $CHCl_3$-CH_3OH-H_2O (65:35:10, vol/vol/vol, lower phase) in the solvent system. The spots on the TLC plates were detected through spraying with 10% (vol/vol) H_2SO_4, followed by heating at 110°C for 5 min.

Analysis of ginsenosides by high performance liquid chromatography (HPLC)

The HPLC analysis of the ginsenosides was performed using an HPLC system (Younglin Co. Ltd, Korea) with a quaternary pump, automatic injector, single wavelength UV detector (model 730D), and Younglin's AutoChro-3000 software for peak identification and integration. The separation was performed on a Prodigy ODS(2) C_{18} column (5 μm, 150 × 4.6 mm i.d.; Phenomenex, USA) with a guard column (Eclipse XDB C_{18}, 5 μm, 12.5 × 4.6 mm i.d.). The mobile phases were A (acetonitrile) and B (water). The gradient elution started with 32% solvent A and 68% solvent B, and was changed according to the following schedule: from 0-8 min, A was increased from 32 to 65%; from 8-12 min, A was increased from 65 to 100%; from 12-15 min, A was constant at 100%; from 15-18 min, A was decreased from 100 to 32%; from 18-18.1 min, A was constant at 32% from 18.1–28 min. The flow rate was 1.0 ml/min, and the detection was performed by monitoring the absorbance at 203 nm, with an injected volume of 25 μl.

Results and Discussion

Phylogenetic analysis of the BglAch sequence

The β-glucosidase gene (*bglAch*) consists of 1,260 bp encoding 419 amino acids with a molecular mass of 45.8 kDa and a

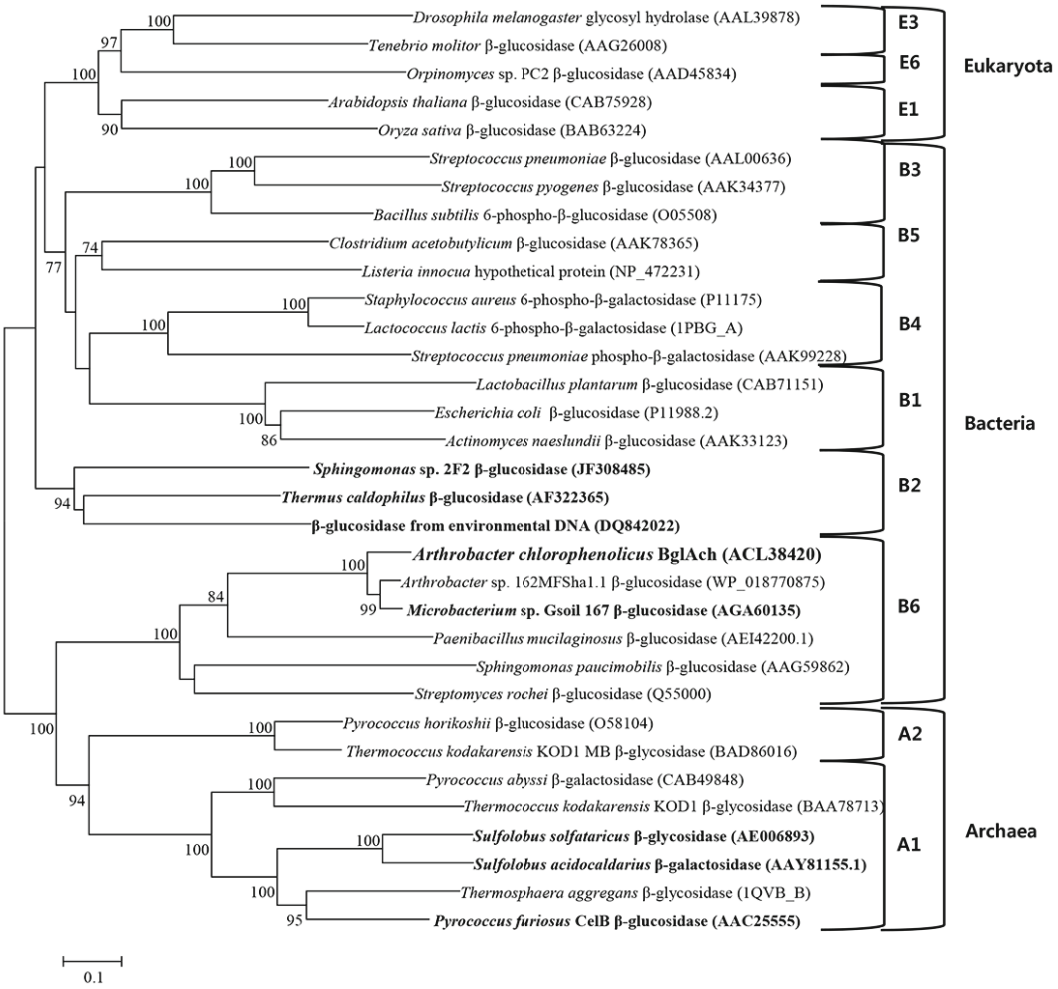

Fig. 1. Phylogenetic analysis of the characterized glycoside hydrolase family 1 (GH1). Amino acid sequences were obtained from the NCBI/EMBL database and CAZY database (accession numbers are indicated on the tree). This tree was made using the neighbor-joining method with a poisson model and pairwise deletion. Bootstrap values expressed as percentages of 1000 replications greater than 65% are shown at the branch points. The bar represents 10 amino acid residue substitutions per 100 amino acid residues. Bold means ginsenoside hydrolyzing *β*-glycosidases.

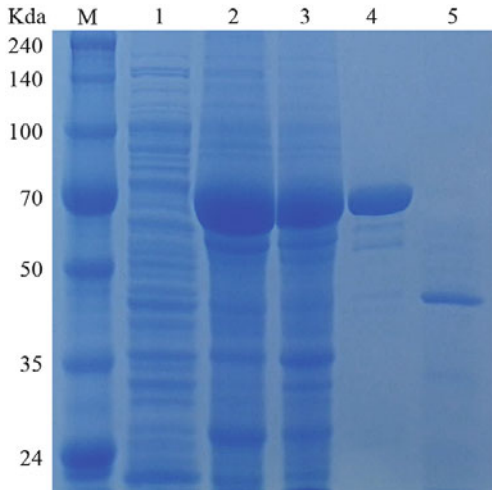

Fig. 2. SDS-PAGE analysis of purified BglAch: 1, uninduced crude extract (5 μl of 20 mg/ml); 2, soluble fraction in crude extract of BL21(DE3) carrying pGEX-BglAch after induction (5 μl of 20 mg/ml); 3, precipitated fraction in crude extract of BL21(DE3) carrying pGEX-BglAch after induction (5 μl of 20 mg/ml); 4, GST-BglAch enzyme fraction after purification by GST·Bind agarose resin (10 μl of 10 mg/ml); 5, purified BglAch (10 μl of 0.2 mg/ml).

theoretical pI value of 4.88 (http://web.expasy.org/compute_pi/). Analysis of the amino acid sequences of BglAch indicated that it was most similar (86.6%) to the glycoside hydrolase of *Arthrobacter* sp. 162MFSha1.1 (GenBank number WP_018770875) followed by that of *Microbacterium* sp. Gsoil 167 (86.2%) (GenBank number AGA60135), which both belong to the glycoside hydrolase family 1 (GH1). Furthermore, the enzymatic activity of the *Microbacterium* sp. Gsoil 167 has been reported to have ginsenoside conversion activity. BglAch has homology to the protein domain of GH1. The CAZY database describes more than 5,000 uncharacterized and 270 characterized GH1 members that are widespread among numerous organisms. Glycosyl hydrolases are classified according to amino acid sequence similarities, which reflect the structural features and substrate specificities of the enzymes from the CAZY database. GH1 has also been subdivided into several subfamilies (Marques *et al.*, 2003). In order to determine the evolutionary position of BglAch within the characterized enzymes in GH1, phylogenetic analysis was conducted using the neighbor-joining method in the MEGA5 Program with bootstrap values based on 1,000 replications. The resulting consensus tree is presented in Fig. 1. BglAch clustered within Bacterial Subfamily 6 (B6) and formed a separate, well-supported clade (bootstrap of 100) with amino acid sequences close to those of both enzymes. Several ginsenoside-hydrolyzing β-glycosidases in GH1 have previously been cloned, including β-glucosidase (BglA) from Environmental DNA (Kim *et al.*, 2007), *Thermus caldophilus* (Son *et al.*, 2008), *Sulfolobus solfataricus* (Noh *et al.*, 2009), *Sulfolobus acidocaldarius* (Noh and Oh, 2009), another β-glucosidase (BglSp) from *Sphingomonas* sp. 2F2 (Wang *et al.*, 2011), and a β-glycosidase from *Pyrococcus furiosus* (Yoo *et al.*, 2011). The relationship between BglAch and these ginsenoside-hydrolyzing β-glucosidases is presented in Fig. 1.

Table 1. Effects of metal ions and chemical agents on the activity of purified BglAch

Metal ions or reagents	Relative activity ± SD (%)	
	1 mM	10 mM
KCl	97.4 ± 4.6	142.2 ± 3.1
$MgCl_2$	93.5 ± 8.9	95.0 ± 2.0
$MnCl_2$	89.9 ± 7.5	109.8 ± 0.4
$CaCl_2$	95.1 ± 5.3	157.8 ± 3.2
$ZnCl_2$	92.7 ±0.9	56.9 ± 0.4
$CoCl_2$	96.2 ± 5.9	105.0 ± 6.0
$CuCl_2$	24.0 ± 1.4	4.5 ± 1.0
$HgCl_2$	18.2 ± 5.3	2.6 ± 1.4
SDS	10.0 ± 0.7	2.7 ± 0.8
EDTA	68.4 ± 0.7	28.5 ± 1.3
β-Mercaptoethanol	102.1 ± 4.9	96.9 ± 12.0
DTT	99.9 ± 3.1	97.8 ± 12.0
Control	100.0 ± 2.7	100.0 ± 6.5

Cloning, expression, and purification of recombinant BglAch

The GST-BglAch fusion gene was expressed in *E. coli* BL21

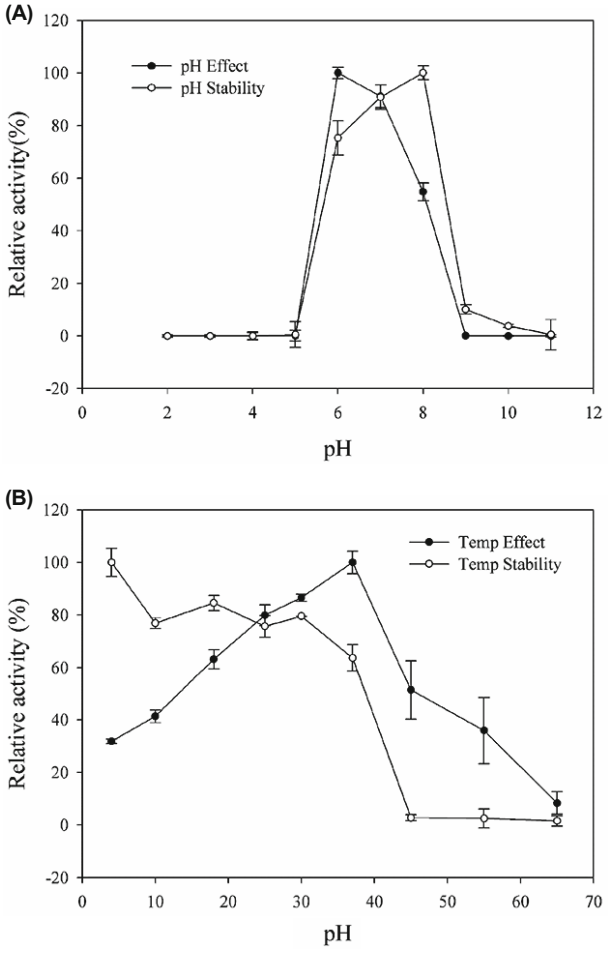

Fig. 3. Effects of pH (A) and temperature (B) on the stability and activity of BglAch.

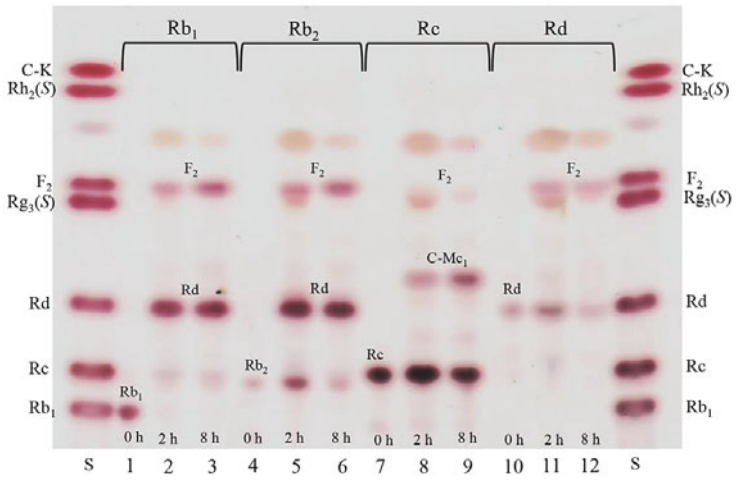

Fig. 4. Thin layer chromatography (TLC) analyses of biotransformation of ginsenosides Rb_1, Rb_2, Rc, and Rd by recombinant BglAch. The sampling times were 2 h and 8 h. Developing solvent: $CHCl_3$-CH_3OH-H_2O (65:35:10, v/v/v, lower phase). S, saponin standards; C-K, compound K; reaction mixture of 1.0 mg/ml Rb_1: 1, 2, 3; reaction mixture of 1.0 mg/ml Rb_2: 4, 5, 6; reaction mixture of 1.0 mg/ml Rc: 7, 8, 9; reaction mixture of 1.0 mg/ml Rd: 10, 11, 12; Reaction time: 1, 4, 7, 10: 0 h; 2, 5, 8, 11: 2 h; 3, 6, 9, 12: 8 h.

(DE3) followed by the induction of 0.1 mM IPTG and incubated at 25°C for 18 h. The GST-BglAch fusion protein was purified using GST·Bind agarose resin and then the GST·Tag sequence was removed by thrombin at room temperature during a 12 h incubation period. Though the predicted molecular mass of the BglAch by amino acid sequences was 45.8 kDa, the estimated molecular weight of BglAch from SDS-PAGE analysis was 43.7 kDa (Fig. 2).

Enzyme characterization

BglAch was active over a narrow pH range (6.0 to 7.0) at 37°C. The optimum pH was 6.0 in sodium phosphate buffer (Fig. 3A). The enzyme lost its optimal activity below pH 5.0 and more than 40% of its optimal activity within 30 min when pH was above 8.0. The *β*-glucosidase from *Paecilo-*

Table 2. Relative activity of the purified recombinant BglAch on various chromogenic substrates as measured by ONP or PNP release at 37°C

Substrate[a]	Relative activity ± SD (%)[b]
PNP-α-D-glucopyranoside	ND[c]
PNP-α-D-mannopyranoside	ND[c]
PNP-α-D-xylopyranoside	ND[c]
PNP-α-L-arabinofuranoside	ND[c]
PNP-α-L-arabinopyranoside	6.6 ± 0.2
PNP-α-L-D-fucopyranoside	ND[c]
PNP-α-L-rhamnopyranoside	ND[c]
PNP-β-D-fucopyranoside	81.2 ± 2.5
PNP-β-D-galactopyranoside	7.5 ± 0.2
PNP-β-D-glucopyranoside	18.4 ± 0.3
PNP-β-D-glucosaminide	ND[c]
PNP-β-D-mannopyranoside	ND[c]
PNP-β-D-xylopyranoside	ND[c]
PNP-β-L-arabinopyranoside	ND[c]
ONP-α-D-galactopyranoside	ND[c]
ONP-β-D-fucopyranoside	100.0 ± 3.1
ONP-β-D-galactopyranoside	18.2 ± 1.2
ONP-β-D-glucopyranoside	55.2 ± 0.3

[a] Final concentration, 2.0 mM
[b] Activity toward ONP-β-D-fucopyranoside was set as 100%.
[c] Not detected.

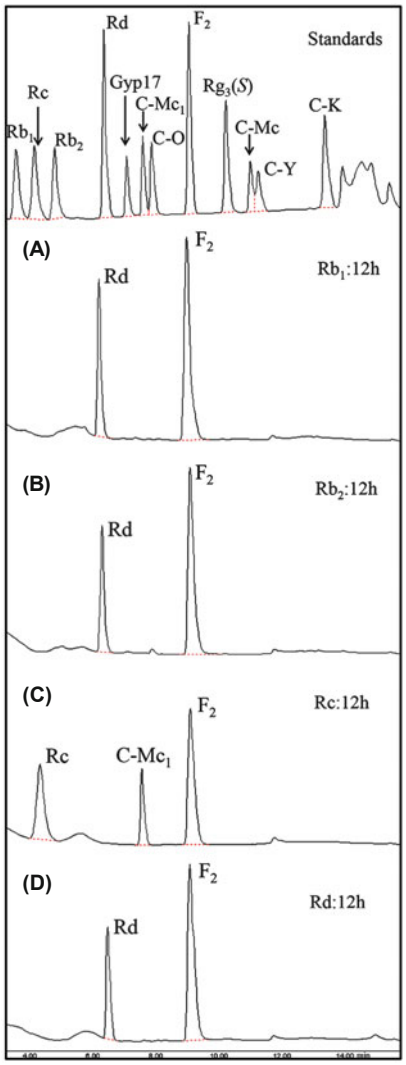

Fig. 5. HPLC results of the transformation of ginsenosides Rb_1, Rb_2, Rc, and Rd by recombinant BglAch. The sampling time was 12 h. (A) biotransformation of ginsenoside Rb_1, (B) Biotransformation of ginsenoside Rb_2, (C) biotransformation of ginsenoside Rc; D, biotransformation of ginsenoside Rd.

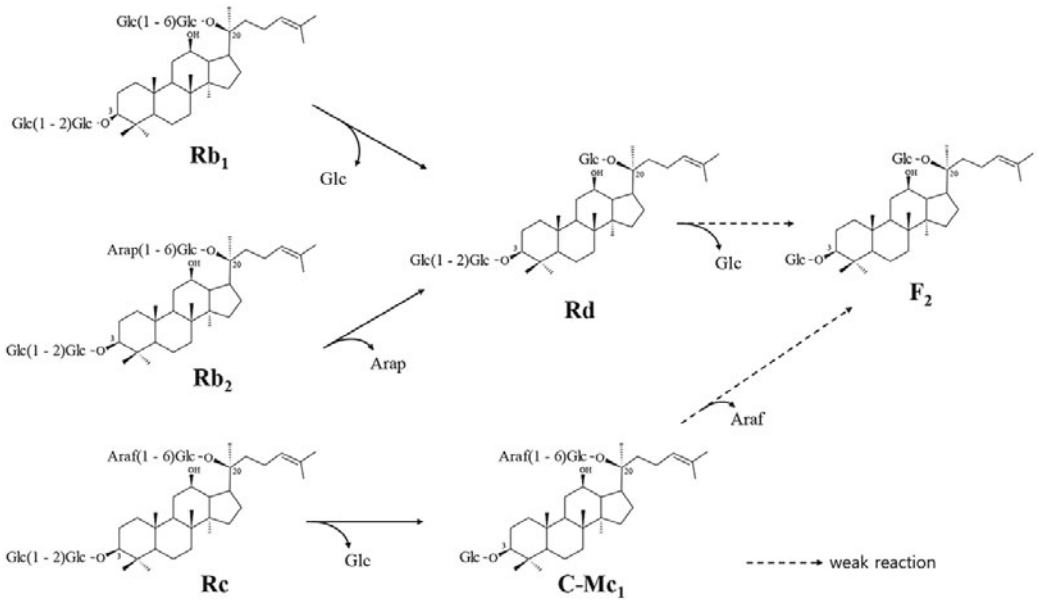

Fig. 6. **Transformation pathways of ginsenosides Rb_1, Rb_2, Rc, and Rd by recombinant BglAch.**

myces Bainier sp. 229 (Yan *et al*., 2008), *Thermus caldophilus* (Son *et al*., 2008), and *Sphingomonas* sp. 2F2 (Wang *et al*., 2011) had optima at pH 3.5, 5.0, and 7.0, respectively. The optimal temperature for BglAch activity was 37°C and the enzyme was stable at lower than 25°C. The enzyme lost 49% of its activity at 45°C, and 65% of its activity at 55°C. It was relatively tolerant to heat when it was derived from a mesophilic strain (Westerberg *et al*., 2000; see Fig. 3B). However, the purified *β*-glucosidase from *Paecilomyces Bainier* sp. 229 (Yan *et al*., 2008), *Thermus caldophilus* (Son *et al*., 2008), and *Sulfolobus solfataricus* (Noh *et al*., 2009) had optimal temperatures of 55, 75, and 90°C, respectively.

The effects of metal ions, EDTA, β-mercaptoethanol, and SDS on BglAch activity were investigated (Table 1). BglAch activity was not affected by DTT or *β*-mercaptoethanol, which is a well-known thiol group inhibitor. K^+ or Ca^{2+} had positive effects on the activity of the enzyme, while enzyme activity was inhibited in the presence of both 1 mM and 10 mM Cu^{2+} or Hg^{2+}. The chelating agent EDTA, and sodium dodecyl-sulfate (SDS), also inhibited BglAch activity.

The substrate specificity of BglAch was tested using 2.0 mM of *p*-nitrophenyl (PNP) and *o*-nitrophenyl (ONP)-glycosides with *α* and *β* configurations (total of 18 kinds), as reported previously (An *et al*., 2010). BglAch was maximally active against ONP-*β*-D-fucopyranoside, followed by PNP-*β*-D-fucopyranoside (81.2% compared to ONP-*β*-D-fucopyranoside), and ONP-*β*-D-glucopyranoside (55.2% compared to ONP-*β*-D-fucopyranoside). Some other substrates, including PNP-*α*-L-arabinopyranoside, PNP-*β*-D-galactopyranoside, PNP-*β*-D-glucopyranoside, and ONP-*β*-D-galactopyranoside; were hydrolyzed a little (Table 1). This explained why BglAch has catalytic activity against ginseno-

Table 3. **Major ginsenoside transformations by the cloned glycoside hydrolase Family 1**

Glycoside hydrolase name	Subfamily	Microorganism	Ginsenoside conversion pathway	Reference
β-Glucosidase (BglAch)	B6	*Arthrobacter chlorophenolicus* $A6^T$	Rb_1→Rd→F_2 Rb_2→Rd→F_2 Rc→C-Mc_1→F_2	This study
β-Glucosidase (BglA)	B2	Environmental DNA	Rb_1→Rd	Kim *et al*. (2007)
β-Glucosidase	B2	*Thermus caldophilus*	Rb_1→Rd	Son *et al*. (2008)
β-Glucosidase	A1	*Sulfolobus solfataricus*	Rb_1→Rd→F_2→C-K, Rb_2→Rd→F_2→C-K, Rc→C-Mc→C-K	Noh *et al*. (2009)
β-Glycosidase	A1	*Sulfolobus acidocaldarius*	Rb_1→Rd→C-K, Rb_2→CY→C-K, Rc→C-Mc	Noh and Oh (2009)
β-Glucosidase (BglSp)	B2	*Sphingomonas* sp. 2F2	Rb1→Gyp XVII→F_2 Rb_2→C-O→F_2 Rc→C-Mc_1→F_2 Rd→F_2	Wang *et al*. (2011)
β-Glycosidase	A1	*Pyrococcus furiosus*	Rb_1→Rd→C-K, Rb_2→Rd→C-K, Rc→Rd→C-K, C-K→PPD	Yoo *et al*. (2011)

side Rb_2, which had an outer arabinose moiety at the C-20 position of the glycone.

The K_m and V_{max} values for the hydrolysis of PNPGlc by BglAch were 3.19 ± 0.4 mM and 20.1 ± 0.84 μmol/min/mg of protein, respectively.

Biotransformation of ginsenosides

In order to verify the bioconversion pathway of the major ginsenosides using GST-BglAch, TLC and HPLC analyses were performed at regular intervals. Based on the R_f values (Fig. 4), it is clear that GST-BglAch could transform 4 kinds of protopanaxadiol type ginsenosides [Rb_1, Rb_2, Rc, and Rd]. The HPLC profiles of the reaction mixture of the four ginsenosides using GST-BglAch, after a 12 h incubation, are shown in Fig. 5. The identities of the transformed ginsenosides were determined by their retention times compared to standard compounds. GST-BglAch demonstrated the variety of ginsenoside hydrolyzing activity. The proposed biotransformation pathway (Fig. 6) for the PPD ginsenosides is: $Rb_1 \rightarrow Rd \rightarrow F_2$, $Rb_2 \rightarrow Rd \rightarrow F_2$, $Rc \rightarrow C\text{-}Mc_1 \rightarrow F_2$, $Rd \rightarrow F_2$. All of the Rb_1 was transformed to Rd in 2 h, but not all of the Rd was transformed to F_2 (Fig. 5A). This conversion speed was faster than that obtained using crude enzyme extracts from *Paecilomyces Bainier* sp. 229-7 (Yan *et al*., 2008) and *Bifidobacterium* sp. SH5 (Chi and Ji, 2005), which converted 1 mg/ml of ginsenoside Rb_1 to Rd within 24 h. The biotransformation of all the Rb_2 to Rd occurred in 12 h, but again, not all the Rd was transformed to F_2 (Fig. 5B). This phenomenon was observed in the transformation of ginsenoside Rd as substrate (Fig. 5D). This means that the recombinant BglAch showed a tendency to hydrolyze the outer glucose or arabinopyranose at the C20 position quickly to produce Rd first, and then to hydrolyze the outer glucose moiety weakly at the C3 position into F_2. Consequently, ginsenoside Rd was not completely transformed to F_2 during longer reaction times. Additionally, BglAch more slowly transformed Rc to F_2 via $C\text{-}Mc_1$ (compared to hydrolysis of Rb1 or Rb_2) (Fig. 5C). Although BglAch did not show any hydrolysis activity to PNP-arabinofuranoside (Table 1), it could bio-transform $C\text{-}Mc_1$ to F_2, cleaving the outer *L*-arabinofuranoside moiety attached at the C20 position. The conversion pathways for Rb_1 Rb_2, Rc, and Rd are unique and have not been reported previously.

β-Glucosidases have been primarily classified in the glycosyl-hydrolase Families 1 and 3, which commonly form closely related subfamilies, with a wide range of activities. Some β-glucosidases in these subfamilies might not have only one specific activity. Furthermore, they might have a high specificity to only glucosides, or glucosides together with arabinosides; while *β*-galactosides, *β*-mannosides, and *β*-xylosides might also be hydrolyzed (Opassiri *et al*., 2006). In addition, many enzymes in this group are able to hydrolyze various types of sugars from aglycones; for example, disaccharide acuminose, malonyl glucose (Chuankhayan *et al*., 2005), and isoflavone glycosides (Kaya *et al*., 2008). When ginsenosides were used as substrate; *β*-glucosidase hydrolyzed the inner and outer glucoses attached to ginsenosides at the C-3 and C-20 positions, and hydrolyzed the outer arabinoside at the C-20 site using various transformation pathways. With regard to GH1, where BglAch is included, there have been several reports on ginsenoside conversion (Table 3). For example, *β*-glycosidase derived from *Pyrococcus furiosus* initially hydrolyzed the outer glucose (*L*-arabinopyranoside and *L*-arabinofuranoside moiety at the C20 position). This was followed by hydrolysis of the inner and outer glucose moieties at the C20 site to make compound K (Yoo *et al*., 2011). The other enzyme [*β*-glucosidase (BglSp) from *Sphingomonas* sp. 2F2] initially converted Rb_1, Rb_2, and Rc into gypenoside XVII, C-O, and $C\text{-}Mc_1$, respectively; by hydrolyzing the outer glucose at the C3 position; then into F_2 by hydrolyzing the C-20 glucose moiety (Wang *et al*., 2011). As a member of GH1, BglAch exhibited a similar ginsenoside-transforming pathway to that of *β*-glucosidase from *Sulfolobus solfataricus* (Noh *et al*., 2009); however, BglAch didn't exhibit $F_2 \rightarrow$ C-K transformation activity.

In conclusion, a recombinant ginsenoside hydrolyzing glycosidase (BglAch), belonging to the GH1 family, was constructed from *Arthrobacter chlorophenolicus* KACC 11414^T in an effort to obtain more effective biotransformation of major ginsenosides. This enzyme was expressed in *E. coli* BL21 (DE3) in a soluble form and was characterized. It had optimum activity at 37°C and pH 6.0. The recombinant BglAch could convert ginsenosides Rb_1, Rb_2, and Rc into F_2 via Rd, Rd, and $C\text{-}Mc_1$, respectively; through selective hydrolysis of the glucose or arabinoside moieties. Therefore, this report proposes a path to improve the functions of the genes of existing organisms and to selectively produce minor ginsenosides.

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References

Akao, T., Kida, H., Kanaoka, M., Hattori, M., and Kobashi, K. 1998. Drug metabolism: intestinal bacterial hydrolysis is required for the appearance of compound K in rat plasma after oral administration of ginsenoside Rb_1 from *Panax ginseng*. *J. Pharm. Pharmacol.* **50**, 1155–1160.

An, D.S., Cui, C.H., Lee, H.G., Wang, L., Kim, S.C., Lee, S.T., Jin, F., Yu, H., Chin, Y.W., Lee, H.K., Im, W.T., and Kim, S.G. 2010. Identification and characterization of a novel *Terrabacter ginsenosidimutans* sp. nov. beta-glucosidase that transforms ginsenoside Rb_1 into the rare gypenosides XVII and LXXV. *Appl. Environ. Microbiol.* **76**, 5827–5836.

Attele, A.S., Wu, J.A., and Yuan, C.S. 1999. Ginseng pharmacology: multiple constituents and multiple actions. *Biochem. Pharmacol.* **58**, 1685–1693.

Chi, H. and Ji, G.E. 2005. Transformation of ginsenosides Rb_1 and Re from *Panax ginseng* by food microorganism. *Biotechnol. Lett.* **27**, 765–771.

Cho, I.H. 2012. Effects of *Panax ginseng* in neurodegenerative diseases. *J. Ginseng Res.* **36**, 342–353.

Choi, J.R., Hong, S.W., Kim, Y., Jang, S.E., Kim, N.J., Han, M.J., and Kim, D.H. 2011. Metabolic activities of ginseng and its

constituents, ginsenoside Rb_1 and Rg_1, by human intestinal microflora. *J. Ginseng. Res.* **35**, 301–307.

Choi, S., Kim, T.W., and Singh, S.V. 2009. Ginsenoside Rh_2-mediated G1 phase cell cycle arrest in human breast cancer cells is caused by p15 Ink4B and p27 Kip1-dependent inhibition of cyclin-dependent kinases. *Pharm. Res.* **26**, 2280–2288.

Christensen, L.P. 2009. Ginsenosides chemistry, biosynthesis, analysis, and potential health effects. *Adv. Food. Nutr. Res.* **55**, 1–99.

Chuankhayan, P., Hua, Y., Svasti, J., Sakdarat, S., Sullivan, P.A., and Ketudat Cairns, J.R. 2005. Purification of an isoflavonoid 7-*O*-beta-apiosyl-glucoside beta-glycosidase and its substrates from *Dalbergia nigrescens* Kurz. *Phytochemistry* **66**, 1880–1889.

Cui, C.H., Kim, S.C., and Im, W.T. 2013a. Characterization of the ginsenoside-transforming recombinant β-glucosidase from *Actinosynnema mirum* and bioconversion of major ginsenosides into minor ginsenosides. *Appl. Microbiol. Biotechnol.* **97**, 649–659.

Cui, C.H., Liu, Q.M., Kim, J.K., Sung, B.H., Kim, S.G., Kim, S.C., and Im, W.T. 2013b. Identification and characterization of a *Mucilaginibacter* sp. strain QM49 β-glucosidase and its use in the production of the pharmaceutically active minor ginsenosides (*S*)-Rh_1 and (*S*)-Rg_2. *Appl. Environ. Microbiol.* **79**, 5788–5798.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783–791.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**, 95–98.

Hong, H., Cui, C.H., Kim, J.K., Jin, F.X., Kim, S.C., and Im, W.T. 2012. Enzymatic biotransformation of ginsenoside Rb_1 and gypenoside XVII into ginsenosides Rd and F_2 by recombinant β-glucosidase from *Flavobacterium johnsoniae*. *J. Ginseng Res.* **36**, 418–424.

Kaya, M., Ito, J., Kotaka, A., Matsumura, K., Bando, H., Sahara, H., Ogino, C., Shibasaki, S., Kuroda, K., Ueda, M., and *et al.* 2008. Isoflavone aglycones production from isoflavone glycosides by display of β-glucosidase from *Aspergillus oryzae* on yeast cell surface. *Appl. Microbiol. Biotechnol.* **79**, 51–60.

Kim, J.K., Cui, C.H., Liu, Q., Yoon, M.H., Kim, S.C., and Im, W.T. 2013. Mass production of the ginsenoside Rg_3(*S*) through the combinative use of two glycoside hydrolases. *Food Chem.* **141**, 1369–1377.

Kim, J.K., Cui, C.H., Yoon, M.H., Kim, S.C., and Im, W.T. 2012. Bioconversion of major ginsenosides Rg_1 to minor ginsenoside F_1 using novel recombinant ginsenoside hydrolyzing glycosidase cloned from *Sanguibacter keddieii* and enzyme characterization. *J. Biotechnol.* **161**, 294–301.

Kim, S.J., Lee, C.M., Kim, M.Y., Yeo, Y.S., Yoon, S.H., Kang, H.C., and Koo, B.S. 2007. Screening and characterization of an enzyme with β-glucosidase activity from environmental DNA. *J. Microbiol. Biotechnol.* **17**, 905–912.

Lee, J.H., Ahn, J.Y., Shin, T.J., Choi, S.H., Lee, B.H., Hwang, S.H., Kang, J., Kim, H.J., Park, C.W., and Nah, S.Y. 2011. Effects of minor ginsenosides, ginsenoside metabolites, and ginsenoside epimers on the growth of *Caenorhabditis elegans*. *J. Ginseng Res.* **35**, 375–383.

Leung, K.W. and Wong, A.S. 2010. Pharmacology of ginsenosides: a literature review. *Chin. Med.* **5**, 20–22.

Mai, T.T., Moon, J., Song, Y., Viet, P.Q., and Phuc, P.V. 2012. Ginsenoside F_2 induces apoptosis accompanied by protective autophagy in breast cancer stem cells. *Cancer Lett.* **321**, 144–153.

Marques, A.R., Coutinho, P.M., Videira, P., Fialho, A.M., and Sa-Correia, I. 2003. *Sphingomonas paucimobilis* β-glucosidase *Bgl*1: a member of a new bacterial subfamily in glycoside hydrolase family 1. *Biochem. J.* **370**, 793–804.

Noh, K.H. and Oh, D.K. 2009. Production of the rare ginsenosides compound K, compound Y, and compound Mc by a thermostable β-glycosidase from *Sulfolobus acidocaldarius*. *Biol. Pharm. Bull.* **32**, 1830–1835.

Noh, K.H., Son, J.W., Kim, H.J., and Oh, D.K. 2009. Ginsenoside compound K production from ginseng root extract by a thermostable β-glycosidase from *Sulfolobus solfataricus*. *Biosci. Biotechnol. Biochem.* **73**, 316–321.

Opassiri, R., Pomthong, B., Onkoksoong, T., Akiyama, T., Esen, A., and Ketudat Cairns, J.R. 2006. Analysis of rice glycosyl hydrolase family 1 and expression of Os4bglu12 β-glucosidase. *BMC Plant Biol.* **6**, 33.

Park, M.W., Ha, J., and Chung, S.H. 2008. 20(*S*)-ginsenoside Rg_3 enhances glucose-stimulated insulin secretion and activates AMPK. *Biol. Pharm. Bull.* **31**, 748–751.

Park, H.J., Kim, D.H., Park, S.J., Kim, J.M., and Ryu, J.H. 2012. Ginseng in traditional herbal prescriptions. *J. Ginseng Res.* **36**, 225–241.

Park, C.S., Yoo, M.H., Noh, K.H., and Oh, D.K. 2010. Biotransformation of ginsenosides by hydrolyzing the sugar moieties of ginsenosides using microbial glycosidases. *Appl. Microbiol. Biotechnol.* **87**, 9–19.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.

Shin, J.Y., Lee, J.M., Shin, H.S., Park, S.Y., Yang, J.E., Cho, S.K., and Yi, T.H. 2012. Anti-cancer effect of ginsenoside F_2 against glioblastoma multiforme in xenograft model in SD rats. *J. Ginseng Res.* **36**, 86–92.

Son, J.W., Kim, H.J., and Oh, D.K. 2008. Ginsenoside Rd production from the major ginsenoside Rb_1 by β-glucosidase from *Thermus caldophilus*. *Biotechnol. Lett.* **30**, 713–716.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739.

Tawab, M.A., Bahr, U., Karas, M., Wurglics, M., and Manfred, S.Z. 2003. Degradation of ginsenosides in humans after oral administration. *Drug Metab. Dispos.* **31**, 1065–1071.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876–4882.

Wang, L., Liu, Q.M., Sung, B.H., An, D.S., Lee, H.G., and Im, W.T. 2011. Bioconversion of ginsenosides Rb_1, Rb_2, Rc and Rd by novel β-glucosidase hydrolyzing outer 3-O glycoside from *Sphingomonas* sp. 2F2: cloning, expression, and enzyme characterization. *J. Biotechnol.* **156**, 125–133.

Westerberg, K., Elvang, A.M., Stackebrandt, E., and Jansson J.K. 2000. *Arthrobacter chlorophenolicus* sp. nov., a new species capable of degrading high concentrations of 4-chlorophenol. *Int. J. Syst. Evol. Microbiol.* **50**, 2083–2092.

Yan, Q., Zhou, W., Li, X.W., Feng, M.Q., and Zhou, P. 2008. Purification method improvement and characterization of a novel ginsenoside-hydrolyzing β-glucosidase from *Paecilomyces Bainier* sp. 229. *Biosci. Biotechnol. Biochem.* **72**, 352–359.

Yoo, M.H., Yeom, S.J., Park, C.S., Lee, K.W., and Oh, D.K. 2011. Production of aglycon protopanaxadiol via compound K by a thermostable β-glycosidase from *Pyrococcus furiosus*. *Appl. Microbiol. Biotechnol.* **89**, 1019–1028.

Yuan, H.D., Kim, S.J., and Chung, S.H. 2011. Beneficial effects of IH-901 on glucose and lipid metabolisms via activating adenosine monophosphate-activated protein kinase and phosphatidylinositol-3 kinase pathways. *Metabolism* **60**, 43–51.