Journal of Biotechnology 156 (2011) 125–133

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

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

Liang Wang[a], Qing-Mei Liu[a], Bong-Hyun Sung[b], Dong-Shan An[c], Hyung-Gwan Lee[a], Song-Gun Kim[c], Sun-Chang Kim[a,d], Sung-Taik Lee[a], Wan-Taek Im[a,d,*]

[a] *Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea*
[b] *Industrial Biotechnology & Bioenergy Research Center, Korea Research Institute of Bioscience & Biotechnology, Daejeon 305-806, Republic of Korea*
[c] *Biological Resource Center, Korea Research Institute of Bioscience & Biotechnology, Daejeon 305-806, Republic of Korea*
[d] *KAIST Institute for Biocentry, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea*

ARTICLE INFO

Article history:
Received 22 June 2011
Received in revised form 13 July 2011
Accepted 19 July 2011
Available online 27 August 2011

Keywords:
Ginsenoside
Bioconversion
β-Glucosidase
Sphingomonas

ABSTRACT

A new β-glucosidase gene (*bglSp*) was cloned from the ginsenoside converting *Sphingomonas* sp. strain 2F2 isolated from the ginseng cultivating filed. The *bglSp* consisted of 1344 bp (447 amino acid residues) with a predicted molecular mass of 49,399 Da. A BLAST search using the *bglSp* sequence revealed significant homology to that of glycoside hydrolase superfamily 1. This enzyme was overexpressed in *Escherichia coli* BL21 (DE3) using a pET21-MBP (TEV) vector system. Overexpressed recombinant enzymes which could convert the ginsenosides Rb_1, Rb_2, Rc and Rd to the more pharmacological active rare ginsenosides gypenoside XVII, ginsenoside C-O, ginsenoside C-Mc_1 and ginsenoside F_2, respectively, were purified by two steps with Amylose-affinity and DEAE-Cellulose chromatography and characterized. The kinetic parameters for β-glucosidase showed the apparent K_m and V_{max} values of 2.9 ± 0.3 mM and 515.4 ± 38.3 μmol min^{-1} mg of protein^{-1} against *p*-nitrophenyl-β-D-glucopyranoside. The enzyme could hydrolyze the outer C3 glucose moieties of ginsenosides Rb_1, Rb_2, Rc and Rd into the rare ginsenosides Gyp XVII, C-O, C-Mc_1 and F_2 quickly at optimal conditions of pH 5.0 and 37 °C. A little ginsenoside F_2 production from ginsenosides Gyp XVII, C-O, and C-Mc_1 was observed for the lengthy enzyme reaction caused by the side ability of the enzyme.

1. Introduction

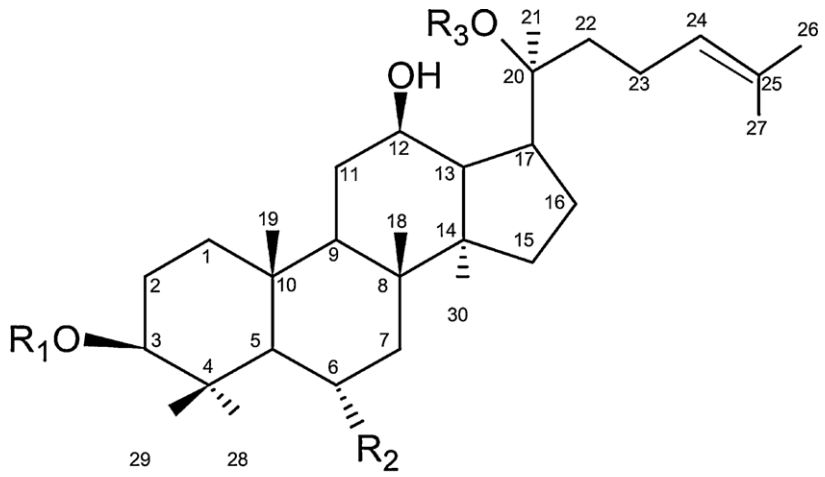
Ginseng, a celebrated traditional herbal product, was used empirically for thousands of years to cure disease and stay healthy. In Asian countries ginseng was deemed to have omnipotent function due to its beneficial effects on the central nervous system, anti-diabetic, anticancer effects and so on (Qi et al., 2011).

Through extensive research and analysis, ginsenosides have come to be regarded as the main constituents responsible for the biological and pharmacological effects of ginseng (Sticher, 1998). Thus far, over 180 kinds of naturally occurring saponins had been isolated (Yoshikawa et al., 1997, 2001, 2003; He et al., 2005; Christensen, 2009; Liu et al., 2010a,b), including malonyl Ra_1, Ra_2, Ra_3, Rb_1, Rb_2, Rc, Rd, Ra_1, Ra_2, Ra_3, Rb_1, Rb_2, Rb_3, Rc, Rd, Re, Rf, Rf_2, Rg_1, Rg_2 (R,S), Rg_3 (R,S), Rg_4, Rg_5, Rg_6, Rh_1 (R,S), Rh_2 (R,S), Rh_3, Rh_4, Rh_{11}, Rh_{12}, Rh_{13}, Rk_1, Rk_2, Rk_3, R_1, R_2, F_1, F_2, F_4, Rs_1, Rs_2, Rs_3 (R,S), Rs_4, Rs_5, Rs_6, Rs_7, Ro, compound K, compound C-Mc_1, compound O, notoginsenoside R_1, quinquenoside R_1 etc. In light of the difference in the structure of the aglycon with a dammarane skeleton (Park et al., 2005), ginsenosides can be categorized into the

Abbreviations: Ginsenoside Rb_1, 3-*O*-[β-D-gluco-pyranosyl-(1–2)-β-D-glucopyranosyl]-20-*O*-[β-D-glucopyranosyl-(1–6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside Rb_2, 3-*O*-[β-D-glucopyranosyl-(1–2)-β-D-glucopyranosyl]-20-*O*-[α-L-arabinopyranosyl-(1–6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside Rc, 3-*O*-[β-D-glucopyranosyl-(1–2)-β-D-glucopyranosyl]-20-*O*-[α-L-arabinofuranosyl-(1–6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside Rd, 3-*O*-[β-D-glucopyranosyl-(1–2)-β-D-glucopyranosyl]-20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol; Gypenoside XVII, 3-*O*-β-D-glucopyranosyl-20-*O*-[β-D-glucopyranosyl-(1–6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside C-O, 3-*O*-β-D-glucopyranosyl-20-*O*-[α-L-arabinopyranosyl-(1–6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside C-Mc_1, 3-*O*-β-D-glucopyranosyl-20-*O*-[α-L-arabinofuranosyl-(1–6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Ginsenoside F_2, 3-*O*-β-D-glucopyranosyl-20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol; Ginsenoside C-K, 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol.

* Corresponding author at: KAIST Institute for Biocentry, KAIST, Daejeon 305-701, Republic of Korea. Tel.: +82 42 3505617; fax: +82 42 3505617.
E-mail address: wandra@kaist.ac.kr (W.-T. Im).

0168-1656/$ – see front matter.
doi:10.1016/j.jbiotec.2011.07.024



Ginsenoside	R_1	R_2	R_3
Rb_1	glc(2→1)glc	H	glc(6→1)glc
Rb_2	glc(2→1)glc	H	glc(6→1)arap
Rc	glc(2→1)glc	H	glc(6→1)araf
Rd	glc(2→1)glc	H	glc
Gyp XVII	glc	H	glc(6→1)glc
Gyp LXXV	H	H	glc(6→1)glc
C-O	glc	H	glc(6→1)arap
C-Y	H	H	glc(6→1)arap
$C\text{-}Mc_1$	glc	H	glc(6→1)araf
C-Mc	H	H	glc(6→1)araf
F_2	glc	H	glc
$20\text{-}Rg_3$	glc(2→1)glc	H	H
$20\text{-}Rh_2$	glc	H	H
C-K	H	H	glc
Re	H	O-glc(2→1)rha	glc
Rg_1	H	O-glc	glc
$20\text{-}Rg_2$	H	O-glc(2→1)rha	H
Rf	H	O-glc(2→1)glc	glc
Rh_1	H	O-glc	H

Fig. 1. Chemical structures of protopanaxadiol and protopanaxatriol ginsenosides. The ginsenosides represented here are all (*S*)-type ginsenosides. glc, β-D-glucopyranosyl; arap, α-L-arabinopyranosyl; araf, α-L-arabinofuranosyl; rha, α-L-rhamnopyranosyl; Gyp, gypenoside; C, compound.

tetracyclic triterpenoid saponins including protopanaxadiol (PPD), protopanaxatriol (PPT), and the pentacyclic triterpenoid saponins including only oleanane ginsenoside Ro. The PPD type ginsenosides were further classified by the position and number of sugar moieties attached by glycosidic bond to the aglycon at the positions of C-3 and C-20 (Fig. 1).

In general, the whole family of ginsenosides exhibits a variety of pharmaceutical functions: immunomodulatory, antitumor, anti-carcinogenic, anti-inflammatory, anti-allergic, anti-atherosclerotic, anti-stress, anti-diabetic, anti-proliferation, anti-genotoxic, and anti-hypertensive as well as neurotransmission modulation effects, etc. (Jia and Zhao, 2009; Sun, 2004; Christensen, 2009). The application of pharmaceutically bioactive minor ginsenosides in industrial and medical areas has created a demand for their availability. However, because of their size, low solubility and poor permeability across the cell membrane, it is difficult for the human body to directly absorb large ginsenosides (Xu et al., 2003), although these components constitute the major portion of the total ginsenoside in raw ginseng. Therefore, production of smaller deglycosylated ginsenosides by the transformation of major ginsenosides is strongly requisite not only for facilitation of the achievement of various types of minor ginsenosides but also for acceleration of researches on the biological and pharmacological properties of minor ginsenosides.

To date, enzymatic and microbiological transformation methods using not only bacteria but also fungi (Liu et al., 2010a,b; Kim et al., 2006; Cheng et al., 2008; Ko et al., 2000) have replaced the traditional acid treatment (Bae et al., 2004) and alkali treatment (Yun et al., 2001) methods for the preparation of ginsenosides because of higher conversion efficiency, fewer by-products, superior environmental protection and better stereo-specificity. Large-scale industrial production of particular ginsenosides is now available by using enzymatic transformation methods.

Herein, we report the cloning of a new gene encoding ginsenoside-transformation β-glucosidase (BglSp) from *Sphingomonas* sp. 2F2, isolated from a ginseng cultivation filed, followed by expression in *Escherichia coli* and characterization of β-glucosidase (BglSp). BglSp belongs to glycoside hydrolase superfamily 1, and the recombinant enzyme employs a selective enzymatic pathway. BglSp could effectively transform ginsenoside Rb_1 → gypenoside XVII and weakly transform XVII → ginsenoside F_2, as well as transform ginsenoside Rb_2 → ginsenoside C-O, ginsenoside Rc → ginsenoside $C\text{-}Mc_1$, and ginsenoside Rd → ginsenoside F_2.

2. Materials and methods

2.1. Bacterial strains, vectors and media

Strain *Sphingomonas* sp. 2F2 with ginsenoside-hydrolyzing β-glucosidase activity was isolated from a ginseng cultivation filed and cultivated on nutrient agar or R2A agar (BD, USA) under aerobic conditions at 30 °C and used for a gene cloning experiment. *E. coli* BL21 (DE3) with pET21-MBP (TEV) vector (Novagen, UK) was used to express MBP fused recombinant enzymes. All *E. coli* cells with plasmids were grown in LB medium (BD, USA) or on LB agar plate at 37 °C supplemented with ampicillin (100 mg/l).

2.2. Chemicals

Ginsenosides Rb_1, Rc, Rb_2, Rd, Rg_1 and Re were purchased from Dalian Green Bio Ltd. (Dalian, China). Ginsenoside F_2 and compound

K were kindly donated by Prof. Jin of the College of Biotechnology, Dalian Polytechnic University, P.R. China. Ginsenosides gypenoside XVII, compound O, compound Mc_1, and compound Mc were obtained in our lab as described by An et al. (2010).

5-Bromo-4-chloro-3-indolyl β-D-glucopyranoside (X-Glc), PNP-β-D-glucopyranoside, PNP-β-D-galactopyranoside, PNP-β-D-fucopyranoside, PNP-N-acetyl-β-D-glucosaminide, PNP-β-L-arabinopyranoside, PNP-β-D-mannopyranoside, PNP-β-D-xylopyranoside, PNP-α-D-glucopyranoside, PNP-α-L-arabinofuranoside, PNP-α-L-arabinopyranoside, PNP-α-L-rhamnopyranoside, PNP-α-D-mannopyranoside, PNP-α-D-xylopyranoside, ONP-β-D-glucopyranoside, ONP-β-D-galactopyranoside, ONP-β-D-fucopyranoside and ONP-α-D-galactopyranoside were obtained from Sigma. The other chemicals used in this study were at least of analytical reagent grade, and the sources are noted individually in Section 2.

2.3. Thin-Layer Chromatography (TLC) analysis

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

2.4. High-Performance Liquid Chromatography (HPLC) analysis

High-Performance Liquid Chromatography (HPLC) analysis of 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 carried out on a ZORBAX Eclipse XDB-C_{18} column (5 μm, 250 mm × 4.6 mm i.d.) (Agilent, USA) with a guard column (Agilent XDB C_{18}, 5 μm, 12.5 mm × 4.6 mm i.d.). The mobile phase was A (acetonitrile) and B (water). Gradient elution started with 25% solvent A and 75% solvent B and was then changed to: A from 25% to 32%, 0–10 min; A from 32% to 55%, 10–15 min; A from 55% to 60%, 15–20 min; A from 60% to 100%, 20–25 min; A 100%, 25–27 min; A from 100% to 25%, 27–30 min; A 25%, 30–40 min. The flow rate was 1.0 ml/min, and detection was performed by monitoring absorbance at 203 nm, with an inject volume of 30 μl.

2.5. Construction of the fosmid library

To clone the ginsenoside β-glucosidase gene from *Sphingomonas* sp. 2F2, a fosmid library was constructed using a CopyControl™ Fosmid Production kit (Epicentre Technologies, WI, USA), according to the manufacturer's protocol. Genomic DNA of *Sphingomonas* sp. 2F2 was randomly sheared into fragments and their size was estimated via long size agarose gel (20 cm × 20 cm) electrophoresis. Afterwards, DNA end-repair was carried out, and the DNA size was selected as 36–40 kb fragments using low melting point (LMP) agar gel electrophoresis. Ligation of the collected fragments into the fosmid vector pCC1FOS was carried out. Fosmid packaging was conducted using MaxPlax™ Lambda-packaging extract. The packaged clones were stored at 4 °C in 1 ml of phage dilution buffer (pH 8.3 10 mM Tris–HCl, 100 mM NaCl, 10 mM $MgCl_2$) against chloroform. The products were transformed into *E. coli* EPI300-$T1^R$ grown in LB broth containing 10 mM $MgSO_4$. Infected *E. coli* was transferred onto LB plates supplemented with 40 μg/ml X-Glc and 12.5 μg/ml chloramphenicol and then incubated at 37 °C for 16 h. The blue color clones were selected as putative β-glucosidase producing clones using sterile toothpicks. After confirmation of TLC assay for ginsenoside-hydrolyzing activity, one clone was selected for fosmid sequencing.

2.6. Fosmid sequencing

Fosmid DNA was purified according to the manufacturer's protocols (Fosmid MAX DNA purification kit, Epicentre, WI). A transposon was inserted into the fosmid clone to facilitate the sequencing procedure. Transposon insertion was performed using a HyperMu™ KAN-1 Insertion Kit (Epicentre, WI). A full fosmid sequence was carried out bidirectionally by two primers which were homologous to the ends of the inserted transposon. The DNA sequencing reactions were performed using an ABI PRISM™ Bigdye™ Terminator chemistry kit and ABI 3730XL capillary DNA Sequencers (Applied Biosystems, Foster City, CA) serviced by Macrogen Co. Ltd. (Korea). The final sequence assembly procedure was conducted using the SeqMan program in the DNASTAR package (DNASTAR, USA), yielding 36.2-kb contig.

2.7. Molecular cloning, expression and purification of recombinant BglSp

The assembled DNA sequence was analyzed using the ORF Finder program on the NCBI website (www.ncbi.nlm.nih.gov/gorf). Predicted ORFs were subjected to a similarity search using BLASTP, which identified a putative open reading frame of a β-glucosidase belonging to glycosyl hydrolase family 1. To clone this gene (*bglSp*), the gene was amplified by PCR using primers containing *Eco*RI and *Hin*dIII restriction sites, respectively, as shown in bold: bglSp F 5′-CG**GAATTC**AGTAATCCGTTCCCCGACGATTTCCTG-3′ and bglSp R 5′-CCC**AAGCTT**CTACCCCAGGCACGCCCCATTGGTC-3′. The amplified fragment was digested with *Eco*RI and *Hin*dIII and inserted into a modified pET-21-(a) vector that contained a tobacco etch virus protease (TEVp) cleavage site and MBP (maltose-binding protein) site. In this case, an MBP-TEV-BglSp fusion protein was constructed.

The resulting recombinant pET-MBP-TEV-*bglSp* was transformed into *E. coli* BL21 (DE3). *E. coli* BL21 (DE3) harboring the recombinant plasmid was grown in LB-ampicillin medium at 37 °C until the culture reached an OD_{600} to 0.6, at which point protein expression was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacterial cells incubated further for 12 h at 18 °C were harvested by centrifugation at 10,000 rpm for 30 min at 4 °C. The cells were washed twice with a solution consisting of 50 mM sodium phosphate, 5 mM EDTA, and 1% Triton X-100 (pH 7.0) and then resuspended in 50 mM sodium phosphate (pH 7.0). The cells were disrupted by ultrasonication (Vibra-cell, Sonics & Materials, CT). Intact cells and debris were removed by centrifugation at 13,000 × rpm for 30 min at 4 °C to obtain a crude cell extract. The MBP-tagged fusion protein was purified by affinity chromatography with an Amylose column (GE Healthcare, USA). The MBP tag was removed by incubation with TEV protease, and the BglSp was further purified by DEAE-cellulose DE-52 chromatography (Whatman). The homogeneity of the protein was assessed by 10% SDS-PAGE and Coomassie Blue staining.

2.8. Enzyme characterization and determination of kinetic parameters

The standard reaction to investigate the specific activity of purified BglSp and the effect of pH, temperature, metals and chemical reagents on the enzyme activity was performed as previously described (An et al., 2010). Substrate preference was examined using chromogenic *o*-nitrophenyl (ONP) and *p*-nitrophenyl (PNP). Kinetic studies were performed with freshly purified enzyme using pNPGlc at concentrations ranging from 0.05 mM to 5 mM. The release of *p*-nitrophenol was immediately measured using a

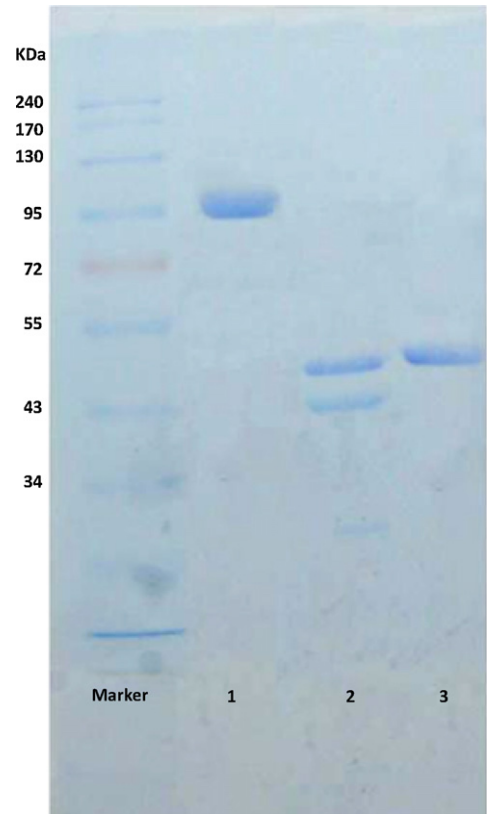


Fig. 2. SDS-PAGE analysis of recombinant BglSp after purification using Amylose and DEAE column. Lanes: 1, MBP-BglSp enzyme fraction after purification by Amylose column chromatography; 2, cleavage of MBP-BglSp by TEV protease; 3, BglSp after DEAE-cellulose chromatography.

microplate reader at 405 nm (Bio-Rad Model 680, Hercules, CA). 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. The parameters (K_m and V_{max}) were determined by an enzyme kinetics program reported by Cleland (1979).

2.9. Enzymatic conversion of ginsenoside Rb_1, Rb_2, Rc, Rd

The initial biotransformation experiments using the major ginsenosides Rb_1, Rb_2, Rc, and Rd as substrates revealed that MBP fused with BglSp does not affect the activities of BglSp protein. Therefore, the fusion protein was used to determine the specificity and selectivity of the enzymes for the hydrolysis of glucose moieties attached at the C-3 and C-20 sites of the four PPD types of ginsenosides. Enzyme solutions at concentrations of 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml in 50 mM sodium phosphate buffer (pH 7.0) 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 sodium phosphate buffer (pH 7.0) at 37 °C. Samples were withdrawn at regular intervals. For TLC analyses, 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 after pretreatment. For 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 sample analyses.

2.10. Nucleotide sequence accession number

The sequences for 16S rRNA and *bglSp* genes from *Sphingomonas* sp. 2F2 were deposited into GenBank/EMBL/DDBJ under the accession numbers JF308484 and JF308485, respectively.

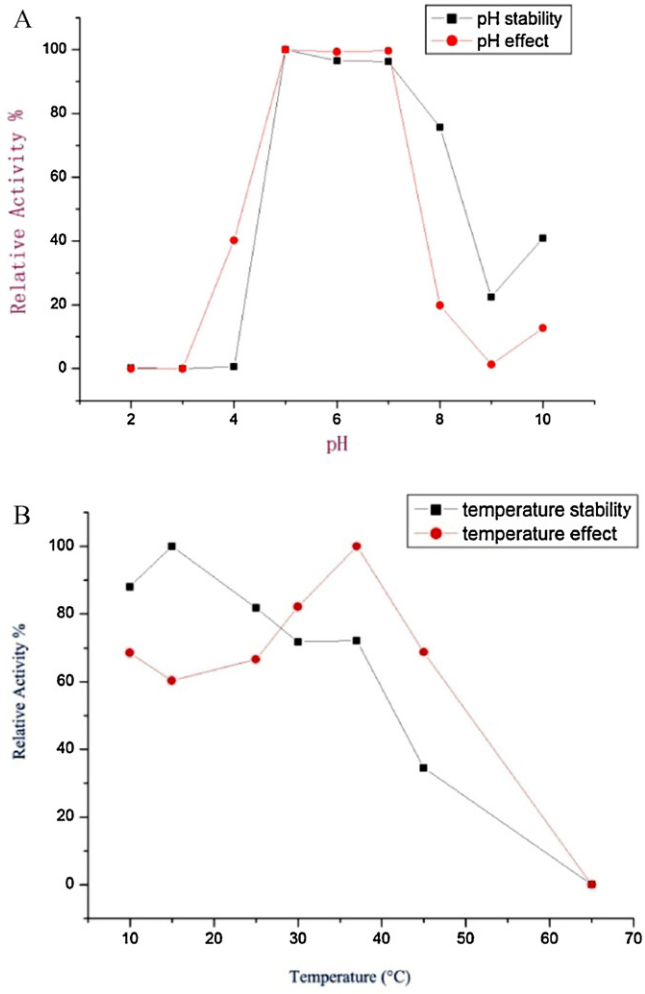


Fig. 3. Effects of pH (A) and temperature (B) on the stability and activity of recombinant BglSp.

3. Results

3.1. Cloning of bglSp and phylogenetic analysis

Genomic DNA from *Sphingomonas* sp. 2F2 was extracted by the phenol–chloroform extraction method and used to generate a fosmid library. The average insert size of the fosmid clone was approximately 40 kb, and the percentage of clones that contained inserts was approximately 90%. Finally, 4 clones out of the 107 clones in the fosmid library turned blue on LB agar plates containing 12.5 μg/ml of chloramphenicol and 40 μg/ml of X-Glc. One of these fosmid clones was positive for the transformation of ginsenosides Rb_1, Rc, and Rd to Gyp XVII, C-Mc_1 and F_2, respectively. This clone was then fully sequenced to determine its identity.

ORF FINDER (www.ncbi.nlm.nih.gov/gorf) analysis of the full sequence of the 36.2 kb clone insert revealed that it encoded a total of 35 putative ORFs (data not shown). One of the ORFs was homologous to glycoside hydrolase genes in glycoside hydrolase family 1. This ORF encoded a gene termed *bgl*Sp that was homologous to the β-glucosidase of *Sphingopyxis alaskensis* RB2256^T of glycoside hydrolase family 1 (73% similarity). In order to determine the evolutionary position of *bglSp* within family 1 glycoside hydrolases, the sequences of all the enzymes produced from bacteria on the website containing the Carbohydrate-Active Enzymes database (CAZy), which is continuously updated for new glycosidase families, were collected and phylogenetic analysis was carried out by the

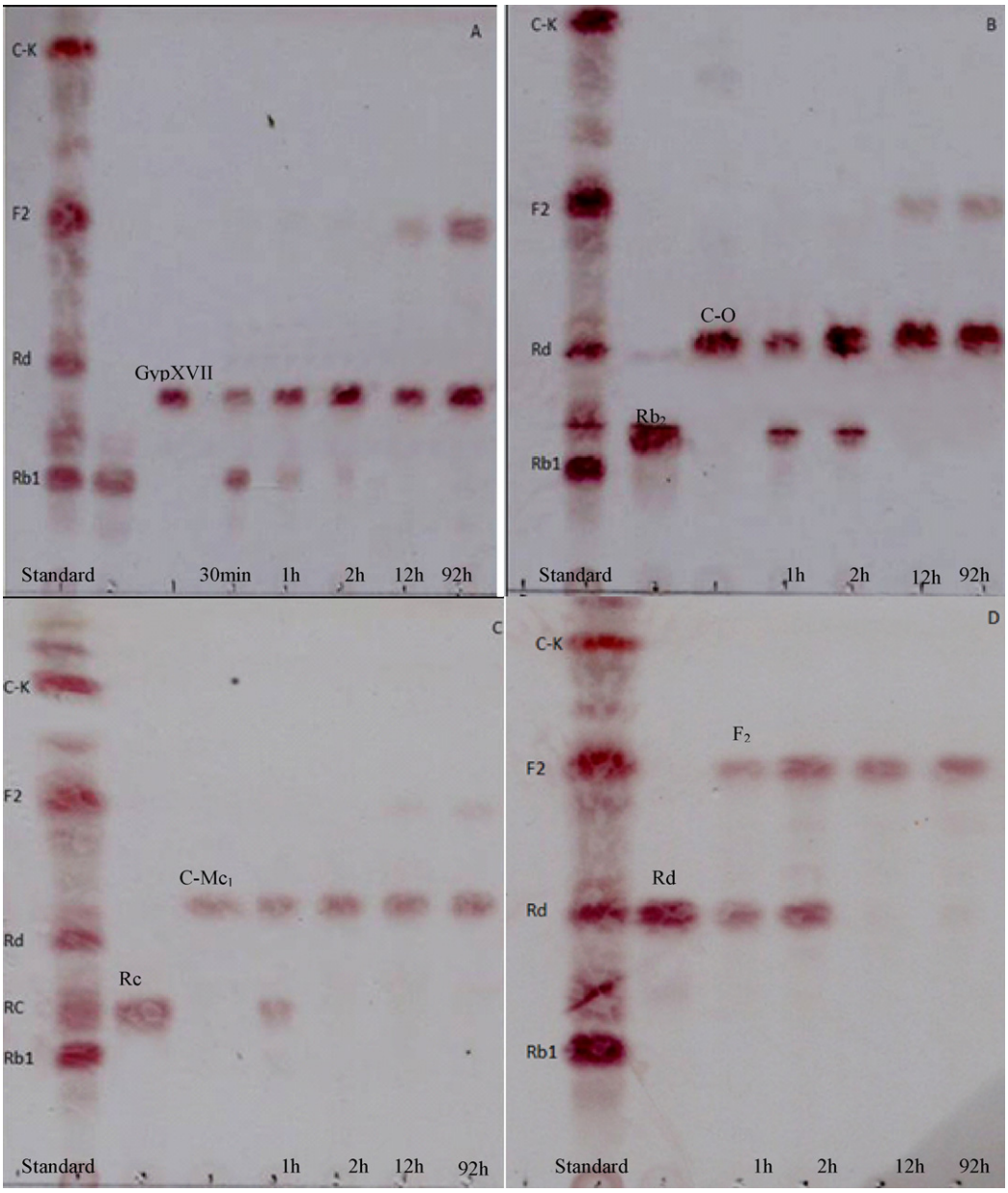


Fig. 4. Time-course Thin Layer Chromatography (TLC) analyses of ginsenoside Rb_1 (A), Rb_2 (B), Rc (C), and Rd (D) by MBP-BglSp at an enzyme concentration of 0.1 mg/ml and ginsenoside concentration of 1.0 mg/ml. Lanes: STD, standard; 0–92 h, reaction time.

neighbor-joining method (Saitou and Nei, 1987) using the MEGA4 Program (Kumar et al., 2007) with bootstrap values based on 1000 replications (Fitch, 1971). The resulting consensus tree is presented in Fig. S1 in the supplementary data.

3.2. Expression and purification of recombinant BglSp

The putative β-glucosidase gene, designated *bglSp*, was amplified by PCR, and then subcloned into the pET21-MBP (TEV) vector to generate the MBP-BglSp fusion gene under the control of an IPTG-inducible promoter, and was expressed in *E. coli* BL21 (DE3). To maximize the yield of the fusion protein in soluble form, we tested different induction conditions and found that induction with 0.1 mM IPTG at 18 °C for 12 h cultivation after induction produced the maximum level of soluble active fusion enzyme (data not shown). The MBP-BglSp fusion protein purified by Amylose-affinity column chromatography was digested by TEV protease to remove the MBP tag at room temperature for 4 h, and the resulting BglSp was further purified by chromatography on DEAE-cellulose, with a single band determined by SDS-PAGE (Fig. 2).

3.3. Enzyme characterization

BglSp was active over a broad pH range (pH 4.0–8.0) in sodium phosphate buffer at 37 °C. The pH activity and stability culminated from pH 5 to 7, where the enzyme kept a nearly stable status for both activity and stability, and from pH 8 the enzyme activity decreased 80%, while at pH 4 the enzyme activity decreased 60% (Fig. 3). The temperature activity culminated at 37 °C, and at 30 °C and 45 °C the enzyme lost 20% and 30% activity, respectively, while no thermostability was determined at 65 °C (Fig. 3). The enzyme activity appeared to be 100% inhibited in the presence of sodium dodecyl-sulfate (SDS) and strongly inhibited in the presence of both 1 mM and 10 mM Hg^{2+} and also inhibited in the presence of 10 mM Cu^{2+}, which caused a 55% activity drop. Mn^{2+} had very little positive effect on the activity of the enzyme; however, no dramatic

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

Metal ions or reagents	Relative activity ± SD (%)	
	1 mM	10 mM
NaCl	101.3 ± 0.8	85.5 ± 0.7
KCl	100.9 ± 1.4	83.6 ± 0. 6
$MgCl_2$	99.1 ± 5.1	89.2 ± 5.6
$MnCl_2$	102.5 ± 1.1	104.6 ± 6.6
$CoCl_2$	100.9 ± 1.5	92.9 ± 4.4
$ZnCl_2$	103.6 ± 0.1	91.9 ± 5.9
$CaCl_2$	101.4 ± 0.6	89.9 ± 6.3
$CuCl_2$	91.1 ± 2.1	45.1 ± 0.9
$HgCl_2$	26.8 ± 0.1	2.4 ± 0.1
SDS	0	0
β-Mercaptoethanol	102.6 ± 1.2	90.8 ± 0.5
DTT	104.8 ± 2.1	91.7 ± 0.8
Control	100 ± 2.3	100 ± 3.2

positive effects on the activity of the BglSp were found for the tested ion (Table 1). The substrate specificity of BglSp was tested using 2.0 mM of *p*-nitrophenyl (pNP) and *o*-nitrophenyl (ONP)-glycosides with α and β configurations. The results, summarized in Table 2, show that BglSp hydrolyzed both pNP-β-D-fucopyranoside and pNP-β-D-glucopyranoside and had a partial hydrolysis effect on pNP-β-D-galactopyranoside. The K_m and V_{max} for the hydrolysis of pNPGlc by β-glycosidase were determined to be 2.9 mM and 515 μmol min^{-1} mg of $protein^{-1}$.

3.4. Biotransformation of major ginsenoside

For the verification of the bioconversion pathway of major ginsenoside by MBP-BglSp, TLC and HPLC analyses were carried out at regular intervals. As shown in Fig. 4, it is clear that MBP-BglSp could transform the four major ginsenosides Rb_1, Rb_2, Rc, and Rd, based on R_f values. As shown in Fig. 5, transformed ginsenosides were determined by their retention times, while standard ginsenosides were determined by HPLC. The final transformed product from ginsenosides Rb_1 and Rd was ginsenoside F_2. MBP-BglSp (same condition as Rb_2, Rc and Rd reaction systems) completely hydrolyzed Rb_1 into gypenoside XVII within 30 min, and only gypenoside XVII was detected during the first hour, while the ginsenoside F_2 appeared after further incubation time, but no further production occurred even after 92 h. Ginsenoside Rd completely converted into F_2 after 12 h and no further production occurred during longer reaction times; this was a valuable metabolism pathway to prepare single F_2 enzymatically. A highly specific transformation act by MBP-BglSp was particularly evident for ginsenosides Rb_2 and Rc. For ginsenoside Rb_2, only ginsenoside C-O was produced during the first 2 h of reaction time, and during longer reaction times, F_2 was produced very weakly, with no progression occurring even after 92 h incubation. For ginsenoside Rc, only C-MC_1 was produced within the first 2 h, and during longer reaction times, little F_2 was produced, and no progression occurred even after 92 h incubation. The enzyme BglSp showed specific hydrolysis activity against the outer C3 glucose moiety of ginsenosides Rb_1, Rb_2, Rc and Rd, which suggests a very useful metabolism pathway for large-scale industrial preparation of minor rare single ginsenosides (Gyp XVII, C-O, C-Mc_1).

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

Substrate	Relative activity ± SD (%)
pNP-α-D-glucopyranoside	0
pNP-α-D-mannopyranoside	0
pNP-α-D-xylopyranoside	0
pNP-α-L-arabinofuranoside	21.1 ± 0.2
pNP-α-L-arabinopyranoside	14.2 ± 0.3
pNP-α-L-rhamnopyranoside	0
pNP-β-D-fucopyranoside	100.6 ± 0.4
pNP-β-D-galactopyranoside	61.1 ± 2.0
pNP-β-D-glucopyranoside	100 ± 1.7
pNP-β-D-glucosaminide	0
pNP-β-D-mannopyranoside	0
pNP-β-D-xylopyranoside	0
pNP-β-L-arabimopyranoside	19.0 ± 0. 7
oNP-α-D-galactopyranoside	0
oNP-β-D-fucopyranoside	23.2 ± 0.1
oNP-β-D-galatopyranoside	0
oNP-β-D-glucopyranoside	35.1 ± 0.4

4. Discussion

Glycoside hydrolases belong to enzyme EC 3.2.1 and can act as catalysts affecting O- or S-glycosides through hydrolysis. Glycoside hydrolases could also be classified as either retaining or inverting enzymes based on the stereochemical function of production by hydrolysis (Sinnott, 1990). Our cloned gene, termed *bglSp*, is homologous to glycoside hydrolase superfamily 1, with amino acid sequence similarity (73%) to the β-glucosidase in *S. alaskensis* RB2256^T. On the phylogenetic tree based on the full-length amino acid sequences shown in Supplementary Fig. S1, BglSp with β-glucosidase produced from *S. alaskensis* RB2256 clustered a separated clade. To the best of our knowledge, the characterized β-glucosidases had been mainly in glycosyl hydrolase families 1 and 3. The enzymes in family 1 commonly formed in a closely related subfamily with a wide range of activities, although they had high sequence similarity. Some β-glucosidases in this subfamily might not have only one specific activity and they might have high specificity to only glucosides or glucosides together with fucosides, while β-galactosides, β-mannosides, and β-xylosides might also be hydrolyzed (Opassiri et al., 2006). In addition, many enzymes in this group were able to hydrolyze various types of sugars from aglycones; for example, disaccharide acuminose and malonyl glucose could be hydrolyzed by isoflavonoid β-glucosidases (Chuankhayan et al., 2005). In fact, one β-glucosidase belonging to family 1 glycoside hydrolases had been cloned from a soil metagenome (Kim et al., 2007). However, the ginsenoside-hydrolysis activity was not investigated and another family 1 thermostable β-glycosidase from *Sulfolobus solfataricus* was also cloned, but the enzyme converted all the Rb_1, Rb_2, and Rc into the final ginsenoside C-K without specical selective conversion activity to the intermidiate stage (Noh et al., 2009). Morever, in case of the type II ginsenosidase, it only hydrolyzed 20-O glycosides of protopanaxadiol type ginsenosides but did not hydrolyze protopanaxatriol type ginsenoside at all described by Yu et al. (2009). This property was totally different from that of our recombinant enzyme. In addition to comparing the previous type I ginsenosidase studied by Yu et al. (2007), our recombinant enzyme had the common point on the ginsenoside-hydrolysis; both of them could hydrolysis only PPD type ginsenosides but not PPT type ginsenosides. However, type I ginsenosidase could hydrolyze the 20-O glucoside of ginsenoside Rb_1, Rb_2, Rc into Rd first followed by hydrolysis of 3-O glucoside of Rd into F_2 and C-K whose property was different from that of our recombinant enzyme. Therefore, BglSp is the first family 1 ginsenoside-converting β-glycosidase hydrolyzing the outer glucose C3 moiety specifically.

Theoretically, each glucose moiety attached to ginsenosides Rb_1, Rb_2, Rc and Rd could be hydrolyzed by β-glucosidase; namely, the outer and inner glucoses attached at positions C-3 and C-20 could be hydrolyzed with various transformation pathways. For example, ($Rb_1 \rightarrow$ Rd or GypXVII, GypXVII $\rightarrow F_2$ or GypLXXV,

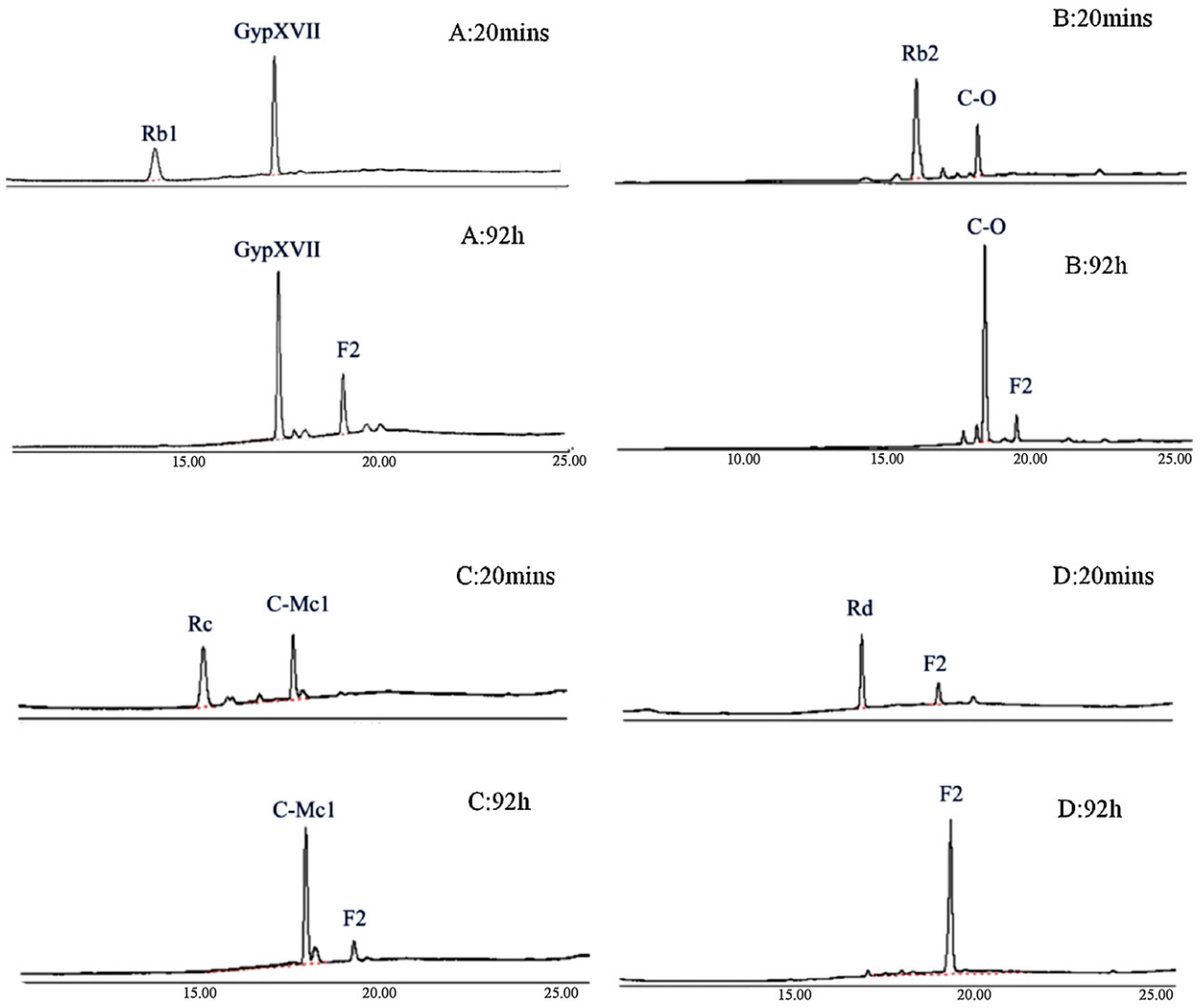


Fig. 5. HPLC results of transformation of ginsenosides Rb_1, Rb_2, Rc and Rd by MBP-BglSp. (A) Ginsenoside Rb_1, (B) ginsenoside Rb_2, (C) ginsenoside Rc, and (D) ginsenoside Rd.

Rd → F_2 or Rg_3). However, in this study, the BglSp could selectively hydrolyze only one outer glucose at position C-3 for major ginsenosides Rb_2, Rc and Rd and could first hydrolyze Rb_1 at position C-3 with a gradual reaction at position C-20. The metabolism pathway could give a direct explanation for the preferential outer glucose reactivity at position C-3 of BglSp: Rb_1 → Gyp XVII; Rb_2 → C-O; Rc → C-Mc_1 and Rd → F_2. For ginsenoside Rb_1 and Rb_2 conversion, in comparison with the same family 1 ginsenoside-hydrolyzing glycosidases, the enzyme preferentially hydrolyzed the outer glucose at position C-20 and did not stop hydrolyzing until the final metabolism ginsenoside C-K was produced, i.e. the ginsenoside-conversion pathway was Rb_1 or Rb_2 → Rd → F_2 → Compound K, which was very different from our selective pathway (Noh et al., 2009). Although Gyp XVII could also be produced by our previous data (An et al., 2010), the Gyp XVII was hydrolyzed stepwise into Gyp LXXV and further into C-K. Other research also showed the ability to ferment Gyp LXXV as an intermediate production by *Acremonium strictum* AS 3.2058 reacted with Rb_1 (Chen et al., 2008), and that with further production F_2, Rg_3 and C-K would appear.

It is very interesting that BglSp contains both strong glucosidase and fucosidase activity, although no data had previously indicated that fucosidase affects the ginsenoside-hydrolysis activity. Notably, arabinofuranosidase and arabinopyranosidase activities were present in BglSp (Table 2), and these two kinds of enzymes characteristics responded to the sugar moieties in the aglycone of Rb_2 and Rc at position C20. This explains the reason for the longer incubation time of Rb2 and Rc with BglSp, since the ginsenoside F_2 would appear from TLC and HPCL data (Figs. 4 and 5). The metabolism pathway for the long reaction time with BglSp would be as follows: Rb_1 → Gyp XVII → F_2; Rb_2 → C-O → F_2; Rc → C-Mc_1 → F_2; Rd → F_2 (Fig. 6).

In conclusion, we have cloned and characterized β-glucosidase of the glycoside hydrolases superfamily 1 from *Sphingomonas* sp. 2F2. Optimal reaction conditions for the enzyme are at 37 °C, pH 5.0. The enzyme has the ability to convert ginsenosides Rb_1, Rb_2, Rc and Rd into rare ginsenosides by selectively hydrolyzing their outer C3 glucose moieties to quickly produce Gyp XVII, C-O, C-Mc_1, and F_2. This is the first report of the cloning of an enzyme that selectively produces Gyp XVII, C-O and C-Mc_1 through the biotransformation of major ginsenosides, and this report offers a path to improve the functions of the existing organism and to selectively produce new alternative ginsenosides.

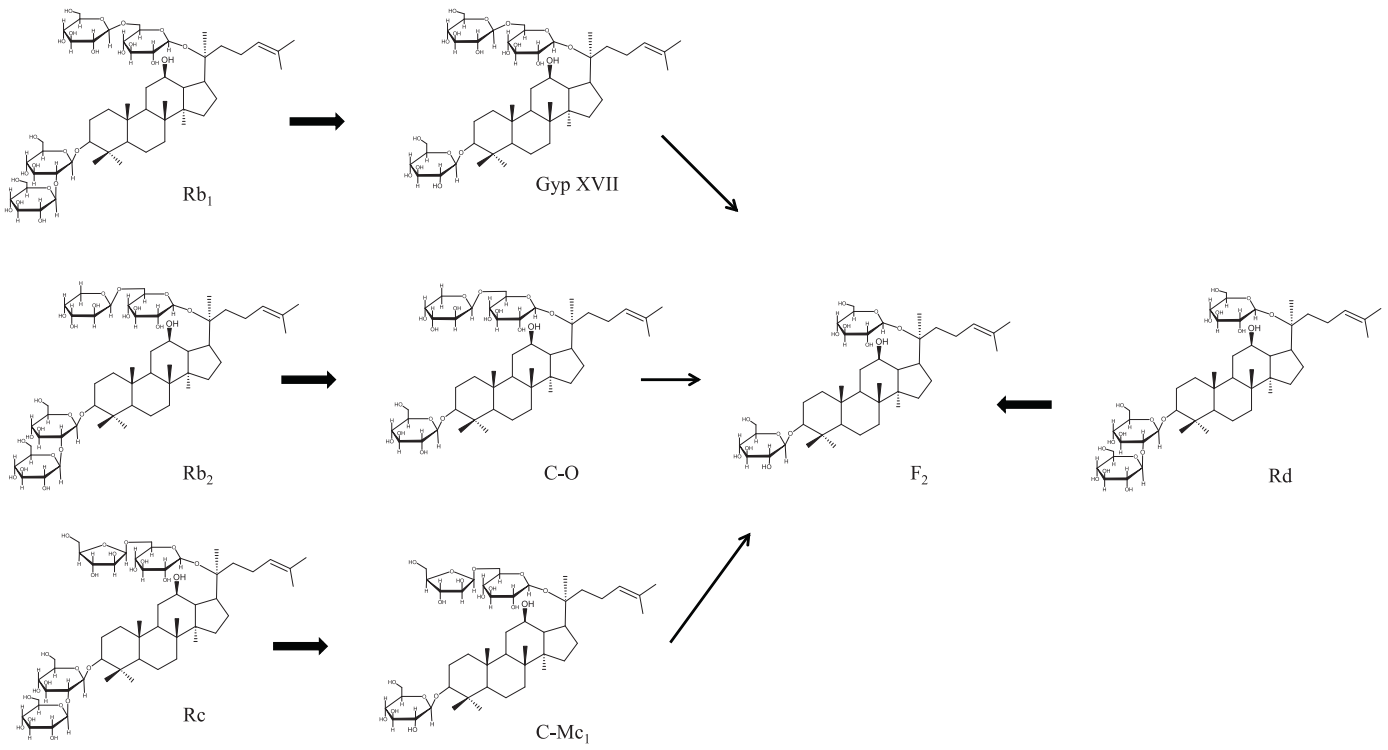


Fig. 6. Transformation pathways from ginsenosides Rb_1, Rb_2, Rc, and Rd to Gyp XVII, C-O, C-Mc_1 and F_2 by recombinant BglSp, respectively.

Acknowledgments

This work was supported by the 21C Frontier Microbial Genomics and Applications Center Program, Ministry of Education, Science & Technology (Grant MG08-0101-2-0) and by the 2011 next generation Biogreen 21 research program of Rural Development Administration, Republic of Korea.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2011.07.024.

References

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., Kim, S.G., 2010. Identification and characterization of a novel *Terrabacter ginsenosidimutans* sp. nov. β-glucosidase that transforms ginsenoside Rb_1 into the rare gypenosides XVII and LXXV. Appl. Environ. Microbiol. 76, 5827–5836.

Bae, E.A., Han, M.J., Kim, E.J., Kim, D.H., 2004. Transformation of ginseng saponins to ginsenoside Rh_2 by acids and human intestinal bacteria and biological activities of their transformants. Arch. Pharm. Res. 27, 61–67.

Chen, G.T., Yang, M., Song, Y., Lu, Z.Q., Zhang, J.Q., Huang, H.L., Wu, L.J., Guo, D.A., 2008. Microbial transformation of ginsenoside Rb_1 by *Acremonium strictum*. Appl. Microbiol. Biotechnol. 77, 1345–1350.

Cheng, L.Q., Na, J.R., Bang, M.H., Kim, M.K., Yang, D.C., 2008. Conversion of major ginsenoside Rb_1 to 20(S)-ginsenoside Rg_3 by *Microbacterium* sp. GS514. Phytochemistry 69, 218–224.

Christensen, L.P., 2009. Chapter 1. Ginsenosides: Chemistry, Biosynthesis, Analysis, and Potential Health Effects.

Chuankhayan, P., Hua, Y., Svasti, J., Sakdarat, S., Sullivan, P.A., 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.

Cleland, W.W., 1979. Statistical analysis of enzyme kinetic data. Methods Enzymol. 63, 103–138.

Fitch, W.M., 1971. Toward defining the course of evolution: minimum change for a specific tree topology. Syst. Biol. 20, 406–416.

He, K., Liu, Y., Yang, Y., Li, P., Yang, L.A., 2005. Dammarane glycoside derived from ginsenoside Rb_3. Chem. Pharm. Bull. (Tokyo) 53, 177–179.

Jia, L., Zhao, Y.Q., 2009. Current evaluation of the millennium phytomedicine – Ginseng (I): etymology, pharmacognosy, phytochemistry, market and regulations. Curr. Med. Chem. 16, 2475–2484.

Kim, B.H., Lee, S.Y., Cho, H.J., You, S.N., Kim, Y.J., Park, Y.M., Lee, J.K., Baik, M.Y., Park, C.S., Ahn, S.C., 2006. Biotransformation of Korean *Panax ginseng* by Pectinex. Biol. Pharm. Bull. 29, 2472–2478.

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

Ko, S.R., Suzuki, Y., Choi, K.J., Kim, Y.H., 2000. Enzymatic preparation of genuine prosapogenin, 20(S)-ginsenoside Rh_1, from ginsenosides Re and Rg_1. Biosci. Biotechnol. Biochem. 64, 2739–2743.

Kumar, S., Dudley, J., Nei, M., Tamura, K., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Liu, G.Y., Li, X.W., Wang, N.B., Zhou, H.Y., Wei, W., Gui, M.Y., Yang, B., Jin, Y.R., 2010a. Three new dammarane-type triterpene saponins from the leaves of *Panax ginseng* C.A. Meyer. J. Asian Nat. Prod. Res. 12, 865–873.

Liu, L., Zhu, X.M., Wang, Q.J., Zhang, D.L., Fang, Z.M., Wang, C.Y., Wang, Z., Sun, B.S., Wu, H., Sung, C.K., 2010b. Enzymatic preparation of 20(S, R)-protopanaxadiol by transformation of 20(S, R)-Rg_3 from black ginseng. Phytochemistry 71, 1514–1520.

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

Opassiri, R., Pomthong, B., Onkoksoong, T., Akiyama, T., Esen, A., Ketudat, Cairns, J.R., 2006. Analysis of rice glycosyl hydrolase family 1 and expression of Os4bglu12 β-glucosidase. BMC Plant Biol. 29, 33.

Park, J.D., Rhee, D.K., Lee, Y.H., 2005. Biological activities and chemistry of saponins from *Panax ginseng* C.A. Meyer. Phytochem. Rev. 4, 159–175.

Qi, L.W., Wang, C.Z., Yuan, C.S., 2011. Ginsenosides from American ginseng: chemical and pharmacological diversity. Phytochemistry 72, 689–699.

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

Sinnott, M.L., 1990. Catalytic mechanisms of enzymatic glycosyl transfer. Chem. Rev. 90, 1171–1202.

Sticher, O., 1998. Getting to the root of ginseng. CHEMTECH 28, 26–32.

Sun, L.Q., 2004. Information on research and application of Ginseng, the king of traditional and herbal medicines. Asian J. Drug Metab. Pharmacokinet. 4, 261–284.

Xu, Q.F., Fang, X.L., Che, D.F., 2003. Pharmacokinetics and bioavailability of ginsenoside Rb_1 and Rg_1 from *Panax notoginseng* in rats. J. Ethnopharmacol. 84, 187–192.

Yoshikawa, M., Morikawa, T., Kashima, Y., Ninomiya, K., Matsuda, H., 2003. Structures of new dammarane-type triterpene saponins from the flower buds of *Panax notoginseng* and hepatoprotective effects of principal ginseng saponins. J. Nat. Prod. 66, 922–927.

Yoshikawa, M., Morikawa, T., Yashiro, K., Murakami, T., Matsuda, H., 2001. Bioactive saponins and glycosides. XIX. Notoginseng (3): immunological adjuvant activity of notoginsenosides and related saponins: structures of notoginsenosides-L, -M, and -N from the roots of *Panax notoginseng* (Burk.) F.H. Chen. Chem. Pharm. Bull. (Tokyo) 49, 1452–1456.

Yoshikawa, M., Murakami, T., Ueno, T., 1997. Bioactive saponins and glycosides. VIII. Notoginseng (1): new dammarane-type triterpene oligoglycosides, notoginsenosides-A, -B, -C, and -D, from the dried root of *Panax notoginseng* (Burk.) F.H. Chen. Chem. Pharm. Bull. (Tokyo) 45, 1039–1045.

Yun, T.-K., Lee, Y.-S., Lee, Y.H., Kim, S.I., Yun, H.Y., 2001. Cancer chemopreventive compounds of red ginseng produced from *Panax ginseng* C.A. Meyer. J. Ginseng Res. 25, 107–111.

Yu, H., Liu, Q., Zhang, C., Lu, M., Fu, Y., Im, W.-T., Lee, S.-T., Jin, F., 2009. A new ginsenosidase from *Aspergillus* strain hydrolyzing 20-O-multi-glycoside of PPD ginsenoside. Process. Biochem. 44, 772–775.

Yu, H., Zhang, C., Lu, M., Sun, F., Fu, Y., Jin, F., 2007. Purification and characterization of new special ginsenosidase hydrolyzing multi-glycosides of protopanaxadiol ginsenosides, ginsenosidase type I. Chem. Pharm. Bull. (Tokyo) 55, 231–235.