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Characterization of the ginsenoside-transforming recombinant β-glucosidase from *Actinosynnema mirum* and bioconversion of major ginsenosides into minor ginsenosides

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Abstract This study focused on the cloning, expression, and characterization of ginsenoside-transforming recombinant β-glucosidase from *Actinosynnema mirum* KACC 20028^T in order to biotransform ginsenosides efficiently. The gene, termed as *bglAm*, encoding a β-glucosidase (BglAm) belonging to the glycoside hydrolase family 3 was cloned. *bglAm* consisted of 1,830 bp (609 amino acid residues) with a predicted molecular mass of 65,277 Da. This enzyme was overexpressed in *Escherichia coli* BL21 (DE3) using a GST-fused pGEX 4T-1 vector system. The recombinant BglAm was purified with a GST·bind agarose resin and characterized. The optimum conditions of the recombinant BglAm were pH 7.0 and 37 °C. BglAm could hydrolyze the outer and inner glucose moieties at the C3 and C20 of the protopanaxadiol-type ginsenosides (i.e., Rb_1 and Rd, gypenoside XVII) to produce protopanaxadiol via gypenoside LXXV, F_2, and $Rh_2(S)$ with various pathways. BglAm can effectively transform the ginsenoside Rb_1 to gypenoside XVII and Rd to F_2; the K_m values of Rb_1 and Rd were 0.69±0.06 and 0.45±0.02 mM, respectively, and the V_{max} values were 16.13±0.29 and 51.56±1.35 μmol min^{-1} mg^{-1} of protein, respectively. Furthermore, BglAm could convert the protopanaxatriol-type ginsenoside Re and Rg_1 into $Rg_2(S)$ and $Rh_1(S)$ hydrolyzing the attached glucose moiety at the C6 and C20 positions, respectively. These various ginsenoside-hydrolyzing pathways of BglAm may assist in producing the minor ginsenosides from abundant major ginsenosides.

Keywords Ginsenoside · Biotransformation · Glycoside hydrolase · *Actinosynnema mirum*

Introduction

In oriental countries, the ginseng root has been used as a traditional medicine for thousands of years and now is widely used around the world (Wang and Yuan 2008). Ginsenosides, also referred to as ginseng saponins, which are isolated from ginseng, have been regarded as the principal constituents responsible for pharmacological activities such as antineoplastic, anti-stress, and antioxidant activities (Attele et al. 1999; Jia and Zhao 2009). More biological activities have been reported on the central nervous system (Nah et al. 2007), cardiovascular system (Zhou et al. 2004), immune system (Jin et al. 2010), and in cancer (Li et al. 2010). More than 180 different ginsenosides have been identified, and they can be categorized as protopanaxadiol (PPD), protopanaxatriol (PPT), and oleanane saponins based on the structure of the aglycon, with a dammarane skeleton (Christensen 2009; Jia and Zhao 2009). The six major ginsenosides (Rb_1, Rb_2, Rc, Rd, Re, and Rg_1) constitute more than 80 % of the total ginsenosides in Korean and American ginseng roots (Qu et al. 2009; Shi et al. 2007), and seven minor ginsenosides (Rg_3, Rh_2, F_2, C-K, Rg_2, Rh_1, and F_1) that are deglycosylated from the major ginsenosides exist in smaller amounts in the ginseng.

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In plants, the glycosylated compounds are often considered as transportable storage compounds or detoxification products that are assumed to lack physiological activity (Sarry and Gunata 2004). This means that the minor ginsenosides may have some chemical reactivity that the major ginsenosides do not. Furthermore, emerging evidence has demonstrated that the minor ginsenosides have more profound pharmaceutical activities, such as anticancer, antidiabetic, antioxidative, and antiaging, than the glycosylated major ginsenosides (Choi et al. 2009, 2011; Kim et al. 2009; Leung and Wong 2010; Park et al. 2008; Qi et al. 2011).

The production of minor ginsenosides from the major ginsenosides has been accomplished through physiochemical methods (Bae et al. 2004; Kim et al. 2000). However, various enzymatic and microbiological methods have been reported thus far for the preparation of minor ginsenosides as a result of their higher conversion efficiency, fewer byproducts, superior environmental protection, and better stereospecificity (Cheng et al. 2008; Kim et al. 2006; Liu et al. 2010). Many ginsenoside-transforming glycoside hydrolases (GHs) have recently been purified from various sources (Park et al. 2010). A classification method has been provided to sort these ginsenoside-hydrolyzing enzymes using transformation pathways. Purified enzymes from *Aspergillus* spp. and *Terrabacter ginsenosidimutans* (An et al. 2010) were characterized and classified into type I, II, III, and IV ginsenosidases (Wang et al. 2011a, 2012; Yu et al. 2007, 2009). This classification helped researchers compare the ginsenoside-transforming activities clearly using transforming pathways, but it could not cover all kinds of pathways because of the diversity of enzyme activities. Neither does it show the correlations between the protein structure and the transforming pathways because of the lack of their sequence information. Thus, the sequenced and recombinant ginsenoside-transforming GHs are higly required to reveal the diverse ginsenoside transformation pathway and have more potential to be used for the production of minor ginsenosides specifically and to be modified for better activities and specificities by mutations.

This study reports on the cloning of a hypothetical gene from *Actinosynnema mirum* KACC 20028^T and the expression of a recombinant β-glucosidase, BglAm, which is characterized as having a ginsenoside-transforming ability. BglAm could hydrolyze major ginsenosides (Rb_1, Rb_2, Rc, Rd, Re, and Rg_1) to minor ginsenosides [Rh_2, compound-O (C-O), compound-Y (C-Y), compound-Mc_1 (C-Mc_1), compound-Mc (C-Mc), compound-K (C-K), gypenoside LXXV (Gyp LXXV), PPD, Rh_1, and PPT] using various cleavage pathways. In these transformations, this is the first report of the biotransformation of Rb_1 into Rh_2 using recombinant enzymes.

Materials and methods

Chemicals

Ginsenosides Rb_1, Rc, Rb_2, Rd, Rg_3(*S*), Rh_2(*S*), Rh_2(*R*), F2, C-K, PPD, Rg_1, Re, Rg_2(*S*), Rh_1(*S*), F_1, and PPT were purchased from Nanjing Zelang Medical Technology Co., Ltd. (China). Ginsenoside gypenoside XVII, gypenoside LXXV, compound O, compound Y, compound Mc_1, and compound Mc were prepared as described by An et al. (2010) and Wang et al. (2011b). 5-Bromo-4-chloro-3-indolyl β-D-glucopyranoside (X-Glc) was obtained from Wako Co., Ltd. (Japan). The other chemicals used in this study were at least of analytical reagent grade, and the sources are noted individually in the "Materials and methods" section.

Bacterial strains, vectors, and media

The strain *A. mirum* KACC 20028^T was purchased from KACC (Suwon, Korea) and cultivated on nutrient agar or R2A agar (BD, USA) under aerobic conditions at 30 °C and used for a gene cloning experiment. *Escherichia coli* BL21 (DE3) with the pGEX 4T-1 plasmid (GE Healthcare) was used to express GST-fused recombinant enzymes. All *E. coli* cells with plasmids were grown in Luria–Bertani (LB) medium at 37 °C and expressed at 18 °C for 12 h supplemented with ampicillin (100 mg/l).

Molecular cloning, expression, and purification of recombinant BglAm

The genomic DNA from *A. mirum* KACC 20028^T was extracted using a genomic DNA extraction kit (Solgent, Daejeon, Korea). The putative β-glucosidase DNA sequence was obtained from whole-genome DNA sequences of *A. mirum* strain 101^T (DSM 43827^T=ATCC 29888^T=NBRC 14064^T=KACC 20028^T; Land et al. 2009). The gene encoding β-glucosidase was amplified from the genomic DNA as a template by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Solgent). The sequence of the oligonucleotide primers used for gene cloning was based on the DNA sequence of *A. mirum* β-glucosidase (GenBank accession no. YP_003100744). Forward (5′-GGT TCC GCG T**GG ATC C**GG TTC CAC CGA CCC CTC CCC TGA C-3′) and reverse primers (5′-GAT GCG GCC G**CT CGA G**TC ATG CCG GCA GGC TAC CTC CCA G-3′) were designed as the primers to introduce the *Bam*HI and *Xho*I restriction sites (underlined), respectively, and were synthesized by Macrogen (Seoul, Korea). The amplified DNA fragment obtained by PCR was purified and then inserted into the pGEX 4T-1 GST fusion vector digested with *Bam*HI and *Xho*I using the EzCloning Kit (Enzynomics

Co., Ltd., Korea). The resulting recombinant pGEX-*bglAm* was transformed into *E. coli* BL21 (DE3). *E. coli* harboring the recombinant plasmid was grown in LB–ampicillin medium at 37 °C until the culture reached an $O.D_{600}$ of 0.6, at which the point relative expression level of soluble BglAm at different conditions was conducted by the addition of different concentrations (0.1, 0.2, and 0.5 mM) of isopropyl-β-D-thiogalactopyranoside (IPTG) at different temperatures (18, 25, and 37 °C). Bacterial cells incubated further for 12 h at 18 °C were harvested by centrifugation at 13,000 rpm for 15 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, USA). Intact cells and debris were removed by centrifugation at 13,000 rpm for 15 min at 4 °C to obtain a crude cell extract. The GST tag was purified by a GST·bind agarose resin. The homogeneity of the protein was assessed using 10 % SDS-PAGE and Coomassie Blue staining.

Enzyme characterization and determination of the kinetic parameters

The standard reaction to investigate the specific activity of purified BglAm 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 0.5 mM chromogenic *o*-nitrophenyl (ONP) and *p*-nitrophenyl (PNP) as substrates at 37 °C for 5 min, with one activity unit being defined as the release of 1 μmol *o*-nitrophenol or *p*-nitrophenol per minute. The following substrates were tested: PNP-β-D-glucopyranoside (PNPGlc), 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 (Sigma).

Kinetic studies were performed with freshly purified enzymes using PNPGlc at concentrations ranging from 0.1 to 5 mM. The release of *p*-nitrophenol was immediately measured using a microplate reader at 405 nm (model 680, Bio-Rad, Hercules, CA). Kinetic studies were performed with freshly purified enzymes using PNPGlc, Rb_1, and Rd at concentrations ranging from 0.1 to 10.0 mM. One unit of activity was defined as the amount of protein required to generate 1 μmol of *p*-nitrophenol or to convert 1 μmol of Rb_1 and Rd 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).

Enzymatic conversion of ginsenosides Rb_1, Rb_2, Rc, Rd, Rg_3, Re, and Rg_1

The initial biotransformation experiments using seven ginsenosides [Rb_1, Rb_2, Rc, Rd, Rg_3(*S*), Re, and Rg_1] as substrates revealed that the GST fused with BglAm does not affect the activities of the BglAm 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 C3 and C20 sites of the seven PPD types of ginsenosides and two PPT types of ginsenosides. Enzyme solutions at a concentration of 1.0 mg/ml in 50 mM sodium phosphate buffer (pH 8.0) were reacted with equal volumes of Rb_1, Rb_2, Rc, Rd, Rg_3(*S*), Re, and Rg_1 solutions at a concentration of 0.1 % (*w*/*v*) in 50 mM phosphate buffer (pH 8.0) at 30 °C. Samples were withdrawn at regular intervals. For thin-layer chromatography (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 high-performance liquid chromatography (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.

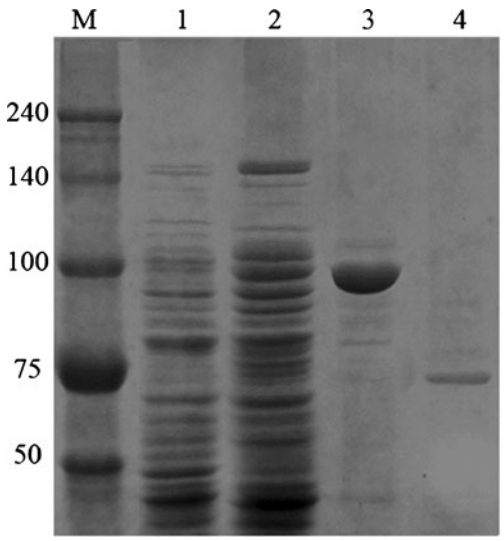

Fig. 1 SDS-PAGE analysis of recombinant BglAm. *Lane M*, molecular weight standard; *lane 1*, crude extract of C41 (DE3) carrying pGEX-BglAm without induction; *lane 2*, soluble fraction in crude extract of BL21 (DE3) carrying pGEX-BglAm after induction; *lane 3*, GST-BglAm enzyme fraction after purification with the GST·bind agarose resin; *lane 4*, purified recombinant BglAm after cleavage by thrombin

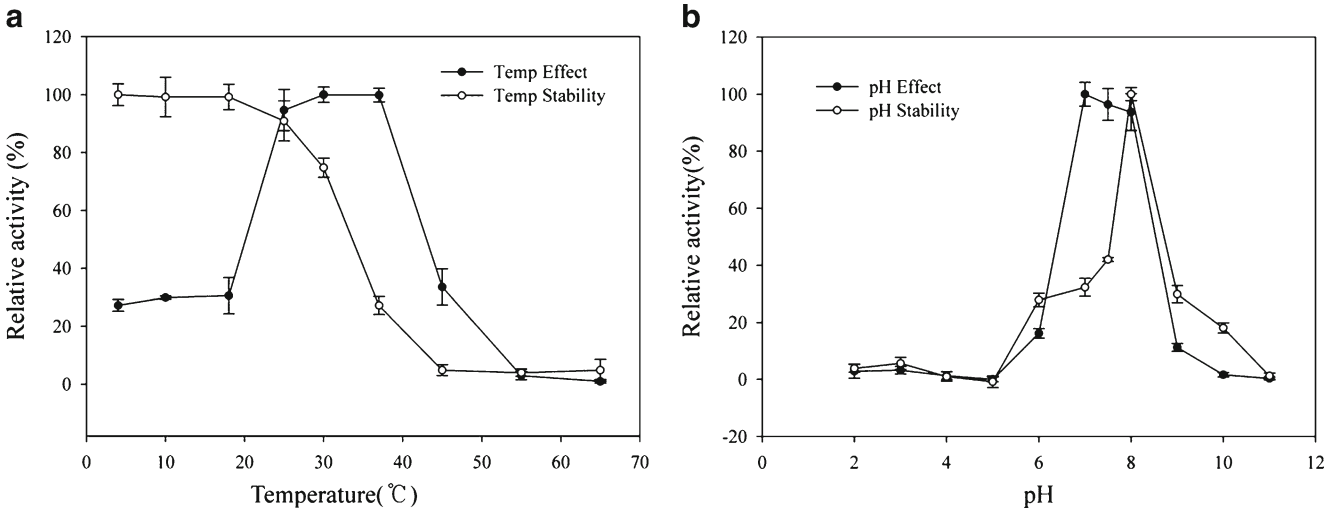

Fig. 2 Effects of temperature (**a**) and pH (**b**) on the stability and activity of recombinant BglAm

Analytical methods of ginsenosides

The TLC analysis was performed using $60F_{254}$ silica gel plates (Merck, Germany) with $CHCl_3$–CH_3OH–H_2O (65:35:10, *v*/*v*, lower phase) as the developing solvent. The spots on the TLC plates were detected by spraying with 10 % (*v*/*v*) H_2SO_4 followed by heating at 110 °C for 5 min. 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 carried out on a Prodigy ODS(2) C_{18} column (5 μm, 150×4.6-mm i.d.; Phenomenex, USA) with a guard column (5 μm, 12.5×4.6-mm i.d.; Eclipse XDB C_{18}). The mobile phases were acetonitrile (A) and water (B). Gradient elution started with 17 % solvent A and 83 % solvent B changed to: A from 17 to 25 %, 12–20 min; A from 25 to 32 %, 20–30 min; A from 32 to 55 %, 30–35 min; A from 55 to 60 %, 35–40 min; A from 60 to 80 %, 40–45 min; A from 80 to 100 %, 45–50 min; A 100 %, 50–54 min; A from 100 to 17 %, 54.0–54.1 min; and A 17 %, 54.1–65 min. The flow rate was 1.0 ml/min; detection was performed by monitoring the absorbance at 203 nm and an injection volume of 25 μl.

Results

Cloning, expression, and purification of recombinant BglAm

The β-glucosidase gene consisting of 1,830 bp encoding 609 amino acids, with homology to the protein domain of the glycoside hydrolase family 3, was amplified via PCR and then inserted into the pGEX 4T-1 vector. In order to maximize the yield of the fusion protein in a soluble form, different induction conditions were tested; it was found that an induction with 0.1 mM IPTG at 18 °C for 12 h of cultivation after induction produced the maximum level of soluble active fusion enzyme. The expression levels of soluble GST-BglAm fusion protein at different temperatures and IPTG induction concentrations were measured with SDS-PAGE (Electronic supplementary

Table 1 Relative activity of the purified recombinant BglAm on various chromogenic substrates as measured by ONP or PNP release at 37 °C

Substrate[a]	Relative activity±SD (%)[b]
PNP-α-D-glucopyranoside	0
PNP-α-D-mannopyranoside	0
PNP-α-D-xylopyranoside	0
PNP-α-L-arabinofuranoside	0
PNP-α-L-arabinopyranoside	5.2±2.8
PNP-α-L-D-fucopyranoside	0
PNP-α-L-rhamnopyranoside	0
PNP-β-D-fucopyranoside	0
PNP-β-D-glactopyranoside	0
PNP-β-D-glucopyranoside	100±4.6
PNP-β-D-glucosaminide	0
PNP-β-D-mannopyranoside	0
PNP-β-D-xylopyranoside	0
PNP-β-L-arabinopyranoside	0
ONP-α-D-galactopyranoside	0
ONP-β-D-fucopyranoside	0
ONP-β-D-galactopyranoside	0
ONP-β-D-glucopyranoside	98.6±4.1

[a] Final concentration, 0.5 mM

[b] Activity toward PNP-β-D-glucopyranoside was set as 100 %

materials (ESM) Fig. S1). The specific activities of the soluble fraction at various induction conditions were tested with the same protein concentration using PNPGlc (ESM Table S1). The GST-BglAm fusion protein was purified using the GST·bind agarose resin, and the GST tag was removed via thrombin at room temperature for 12 h of incubation. The predictive molecular mass (65.3 kDa) of the native β-glucosidase was determined via SDS-PAGE (Fig. 1).

Enzyme characterization

The recombinant BglAm had an optimal pH activity at 7.0 and stability at pH 8.0 in a sodium phosphate buffer at 37 °C. From pH 9.0, the enzyme activity decreased, while at pH 6.0 the enzyme activity decreased 70 %; no activity was seen when the pH was <4.0 or higher than 10.0 (Fig. 2b). The optimal temperature for the BglAm activity was 37 °C (Fig. 2a), which was lower than the optimum temperatures for the β-glucosidase isolated from *T. ginsenosidimutans* (45 °C; An et al. 2010) and from *Pyrococcus furiosus* (55 °C; Yoo et al. 2011). The enzyme was stable at temperatures lower than 18 °C, and approximately 25 % of the activity was lost after incubation at 37 °C for 120 min. Although the most activated pH and temperature of BglAm were 7.0 and 37 °C, respectively, the enzyme stability decreases quickly in these conditions; thus, a longer reaction time would be preferable at pH 8.0 and 25 °C.

The substrate specificity of BglAm was tested using 0.5 mM PNP and ONP glycosides with both α- and β-configurations. The results, which are summarized in

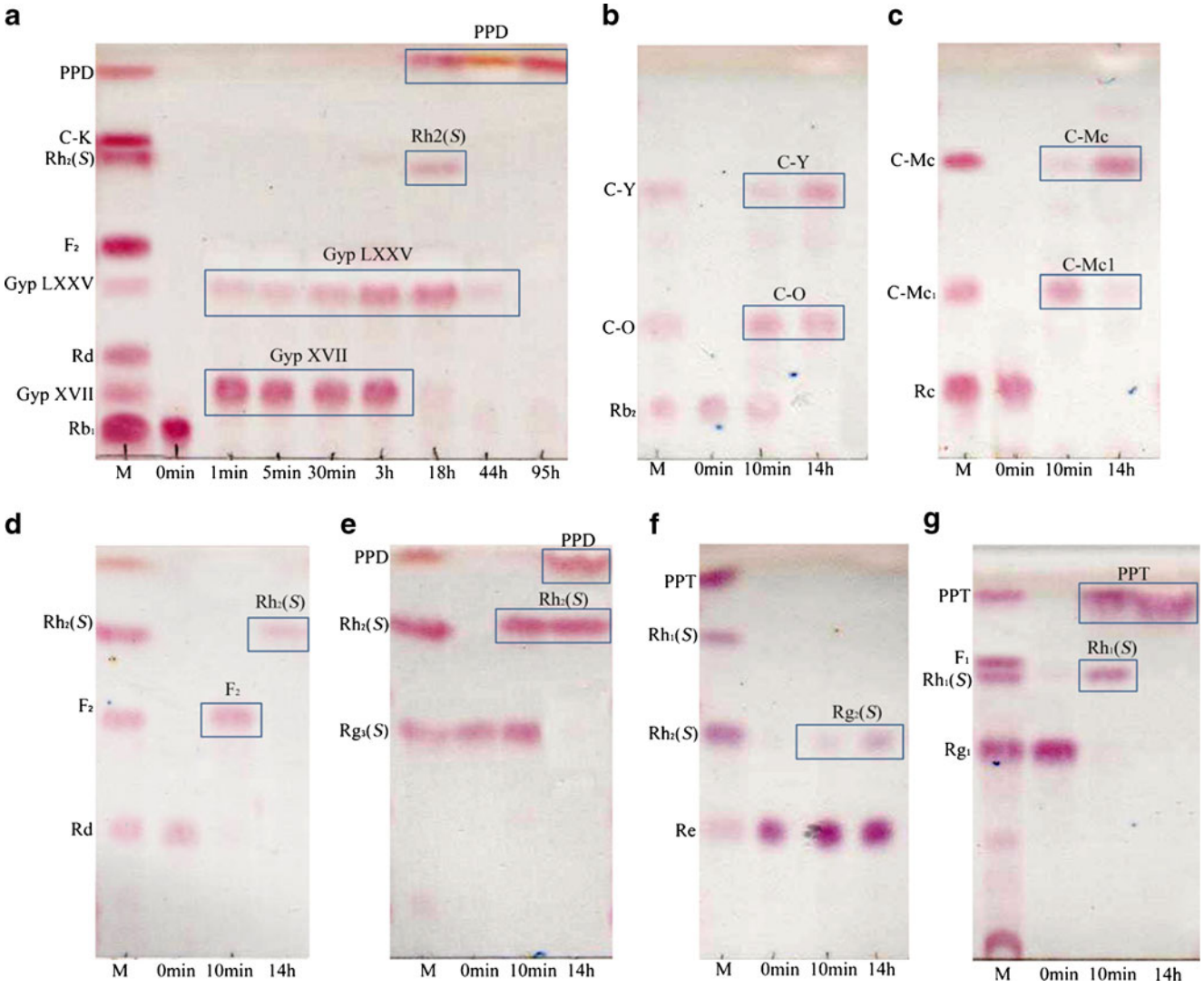

Fig. 3 TLC analyses of the time course of ginsenoside bioconversion by BglAm at an enzyme concentration of 1.0 mg/ml. **a** Transformation of ginsenoside Rb_1. **b** Transformation of ginsenoside Rb_2. **c** Transformation of ginsenoside Rc. **d** Transformation of ginsenoside Rd. **e** Transformation of ginsenoside $Rg_3(S)$. **f** Transformation of ginsenoside Re. **g** Transformation of ginsenoside Rg_1. Developing solvent: $CHCl_3$–CH_3OH–H_2O (65:35:10, *v*/*v*, lower phase). *Lane M*, ginsenoside standards (PPD-type ginsenoside mixtures)

Table 1, demonstrate that BglAm was highly active for the ONP-β-D-glucopyranoside and PNP-β-D-glucopyranoside, and it has a residual activity for PNP-α-L-arabinopyranoside.

The K_m and V_{max} values for the hydrolysis of PNPGlc by BglAm were 0.33±0.01 mM and 5.94±0.06 μmol min^{-1} mg^{-1} of protein, respectively. Furthermore, the K_m and V_{max} values for the biotransformation of Rb_1 to Gyp XVII using GST-BglAm were determined to be 0.69±0.06 mM and 16.13±0.29 μmol min^{-1} mg^{-1} of protein, respectively. The K_m and V_{max} values for the biotransformation of Rd to F_2 were determined to be 0.45±0.02 mM and 51.56±1.35 μmol min^{-1} mg^{-1} of protein, respectively.

Biotransformation pathways of ginsenosides by BglAm

In order to verify the bioconversion pathway of the major ginsenosides using GST-BglAm, TLC and HPLC analyses were performed at regular intervals. As shown Fig. 3, it is clear that GST-BglAm could transform the seven ginsenosides—Rb_1, Rb_2, Rc, Rd, $Rg_3(S)$, Re, and Rg_1—based on the R_f values. For the Rb_1 biotransformation, four types of metabolites were detected as time passed (Fig. 3a). Because Rb_1 has four glucose moieties, it could be hydrolyzed at the inner and outer C3 and C20 aglycon positions. All of Rb_1 were transformed to Gyp XVII in 1 min and consequently transformed to Gyp LXXV. After that, Gyp XVII was transformed completely by 18 h. After 18 h, two bands corresponding to Rh_2 and PPD appeared based on the R_f value. These three bands were proven by HPLC (b in Fig. 4). After 95 h, the ginsenosides to Gyp LXXV and $Rh_2(S)$ were finally converted to PPD. As ginsenoside $Rh_2(S)$ cannot be produced from Gyp LXXV, it is inferred that some Gyp XVII was transformed to $Rh_2(S)$ via cleavage of the inner glucose moiety at the C20 position. This means that the recombinant BglAm preferred the quick hydrolyzation of the outer glucose at position C3 first, and then the inner glucose moiety of C3 is hydrolyzed to Gyp LXXV or the inner glucose moiety of the C20 position removed the two glucose moieties simultaneously. Eventually, the proposed biotransformation pathways for the ginsenoside Rb_1

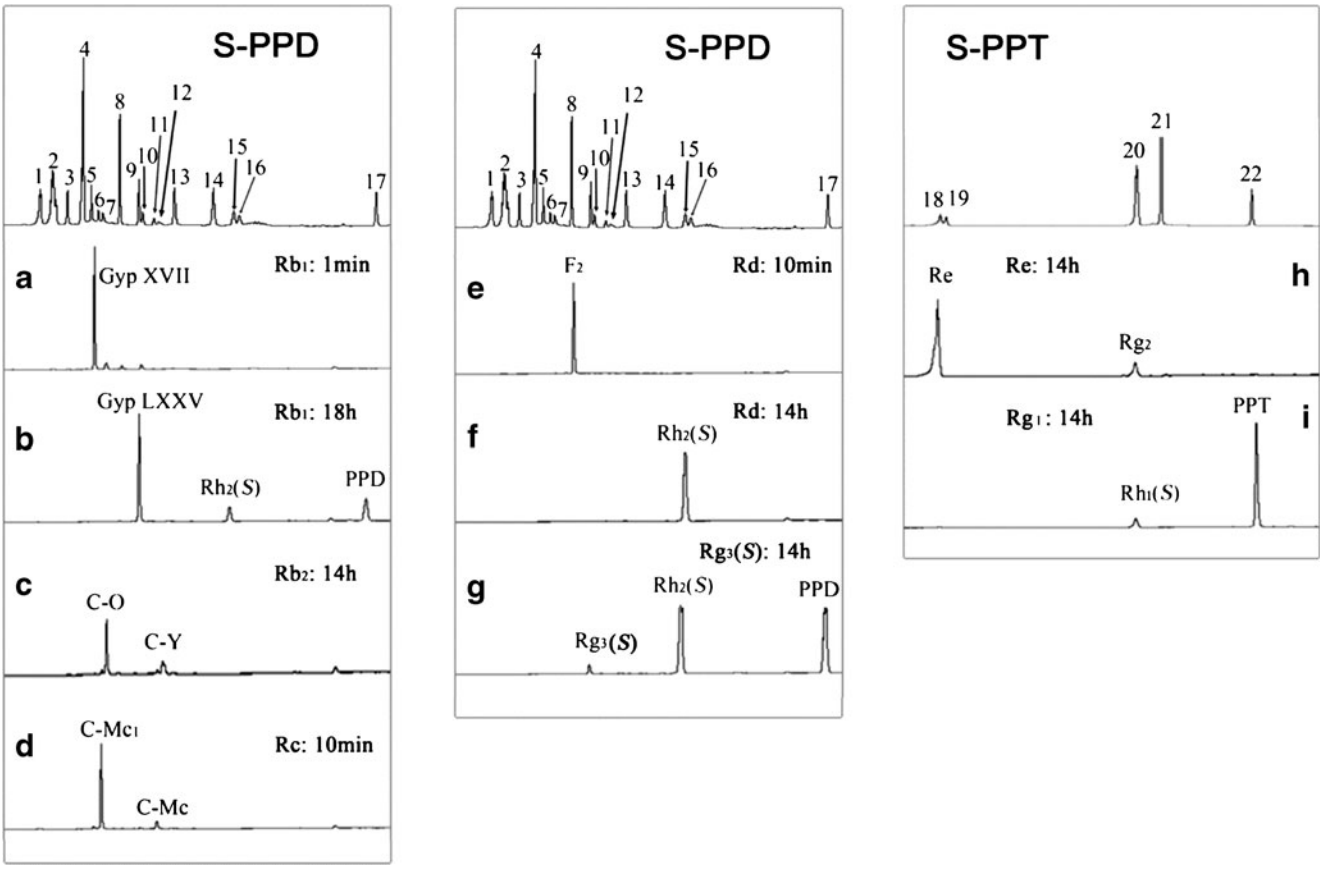

Fig. 4 HPLC results of the transformation of seven kinds of ginsenosides by recombinant BglAm. *a*, *b* Time course of the biotransformation of ginsenoside Rb_1. *c* Biotransformation of ginsenoside Rb_2. *d* Biotransformation of ginsenoside Rc. *e*, *f* Time course of the biotransformation of ginsenoside Rd. *g* Biotransformation of ginsenoside $Rg_3(S)$. *h* Biotransformation of ginsenoside Re. *i* Biotransformation of ginsenoside Rg_1. *S-PPD* and *S-PPT* are the ginsenoside standard peaks: *1* Rb_1, *2* Rc, *3* Rb_2, *4* Rd, *5* Gyp XVII, *6* C-Mc_1, *7* C-O, *8* F_2, *9* $Rg_3(S)$+Gyp LXXV, *10* $Rg_3(R)$, *11* C-Mc, *12* C-Y, *13* PPT, *14* C-K, *15* Rh_2(S), *16* Rh_2(R), *17* PPD, *18* Rg_1, *19* Re, *20* $Rg_2(S)$+$Rh_1(S)$, *21* F_1, *22* PPT

using BglAm are as follows: $Rb_1 \rightarrow$Gyp XVII$\rightarrow$Gyp LXXV and $Rh_2(S) \rightarrow$PPD (a in Fig. 5).

Besides the ginsenoside Rb_1 transformation, BglAm transformed Rb_2 and Rc to C-Y and C-Mc via C-O and C-Mc_1, respectively, through consecutive cleavage of the outer and inner glucose moieties at the C3 position (Fig. 3b, c, and c and d in Fig. 4). However, BglAm could not further hydrolyze C-Y and C-Mc due to the L-arabinopyranoside and L-arabinofuranoside moiety attached at the C20 site. This activity was caused by the very specific reactivities for the substrate

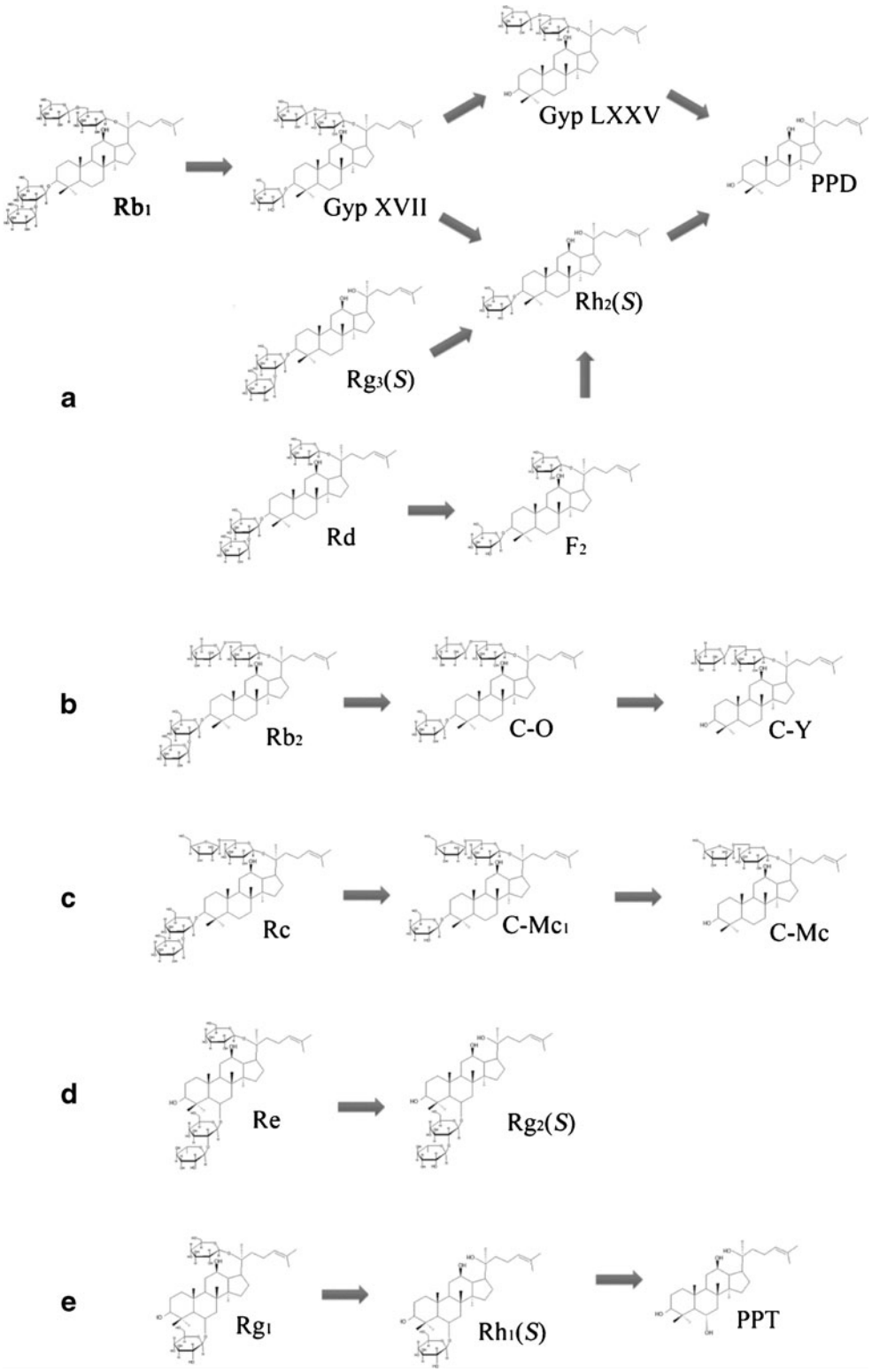

Fig. 5 Transformation pathways of ginsenosides Rb_1, Rd, Rg_3, Rb_2, Rc, Re, and Rg_1 by recombinant BglAm, respectively

preferences of BglAm, as shown in Table 1. The ginsenoside Rd was converted to $Rh_2(S)$ via F_2, via cleavage of the outer glucose at the C3 position first and then cleavage of the inner glucose at the C20 position (Fig. 3d and e in Fig. 4). When the ginsenoside $Rg_3(S)$ was used as a substrate, BglAm hydrolyzed the outer glucose and inner glucose at the C3 position of $Rg_3(S)$ to form PPD via $Rh_2(S)$ (Fig. 3e and f in Fig. 4). These results indicate that BglAm prefers the outer glucose at position C3, followed by the inner glucose at position C3 or C20. The conversion pathways for Rb_1 Rb_2, Rc, and Rd are unique and have not been reported previously. However, the pathway for $Rg_3(S)$ is the same as that of BgpA from *T. ginsenosidimutans* Gsoil 3082^T (An et al. 2010), which is attributed to the high amino acid sequence similarity (68.3 %).

Furthermore, in order to investigate whether BglAm has the specificity and selectivity for the glycone residues attached at the C6 and C20 positions of the PPT ginsenosides, the Re and Rg_1 ginsenosides were also used as substrates. Re was converted to $Rg_2(S)$ weakly through cleavage of the glucose at the C20 position (Fig. 3f and h in Fig. 4). The

Table 2 Major ginsenoside transformations by the cloned glycoside hydrolases

Glycoside hydrolase name	Glycoside hydrolase family	Microorganism	Ginsenoside conversion pathway	Reference
BglA	1	Environmental DNA	$Rb_1 \rightarrow Rd$	Kim et al. (2007)
β-Glucosidase	1	*Thermus caldophilus*	$Rb_1 \rightarrow Rd$	Son et al. (2008)
β-Glucosidase	1	*Sulfolobus solfataricus*	$Rb_1 \rightarrow Rd \rightarrow F_2 \rightarrow C\text{-}K$ $Rb_2 \rightarrow Rd \rightarrow F_2 \rightarrow C\text{-}K$ $Rc \rightarrow C\text{-}Mc \rightarrow C\text{-}K$	Noh et al. (2009)
β-Glycosidase	1	*Sulfolobus acidocaldarius*	$Rb_1 \rightarrow Rd \rightarrow C\text{-}K$ $Rb_2 \rightarrow C\text{-}Y \rightarrow C\text{-}K$ $Rc \rightarrow C\text{-}Mc$	Noh and Oh (2009)
BglSp	1	*Sphingomonas*	Rb1 → Gyp XVII → F2	Wang et al. (2011)
β-Glycosidase	1	*Pyrococcus furiosus*	$Rb_1 \rightarrow Rd \rightarrow C\text{-}K$ $Rb_2 \rightarrow Rd \rightarrow C\text{-}K$ $Rc \rightarrow Rd \rightarrow C\text{-}K$ $C\text{-}K \rightarrow PPD$	Yoo et al. (2011)
rApy-H1	3	*Bifidobacterium longum* H-1	$Rb_2 \rightarrow Rd$	Lee et al. (2011)
BgpA	3	*Terrabacter ginsenosidimutans*	$Rb_1 \rightarrow$ Gyp XVII → Gyp LXXV $Rb_2 \rightarrow C\text{-}O \rightarrow C\text{-}Y$ $Rc \rightarrow C\text{-}Mc_1 \rightarrow C\text{-}Mc$ $Rd \rightarrow F_2 \rightarrow C\text{-}K$ $Rg_3 \rightarrow Rh_2$	An et al. (2010); Jin et al. (2012)
Bgp1	3	*Microbacterium esteraromaticum*	$Rb_1 \rightarrow Rg_3(S)$ $Rd \rightarrow Rg_3(S)$ $Rg_1 \rightarrow Rh_1$ $Re \rightarrow Rg_2$	Quan et al. (2012a, b)
BGL1	3	*Aspergillus niger*	$Rf \rightarrow Rh_1$	Ruan et al. (2009)
BglAm	3	*Actinosynnema mirum*	$Rb_1 \rightarrow$ Gyp XVII → Gyp LXXV → PPD $Rb_1 \rightarrow$ Gyp XVII → $Rh_2(S) \rightarrow$ PPD $Rb_2 \rightarrow C\text{-}O \rightarrow C\text{-}Y$ $Rc \rightarrow C\text{-}Mc_1 \rightarrow C\text{-}Mc$ $Rd \rightarrow F_2 \rightarrow Rh_2(S)$ $Rg_3(S) \rightarrow Rh_2(S) \rightarrow PPD$ $Re \rightarrow Rg_2(S)$ $Rg_1 \rightarrow Rh_1(S) \rightarrow PPT$	This study
rAfuB-H1	51	*Bifidobacterium longum* H-1	$Rc \rightarrow Rd$	Lee et al. (2011)
AbfA	51	*Rhodanobacter ginsenosidimutans* Gsoil 3054	$Rc \rightarrow Rd$ $C\text{-}Mc_1 \rightarrow F_2$ $C\text{-}Mc \rightarrow C\text{-}K$	An et al. (2012)

ginsenoside Rg_1 was converted to PPT via $Rh_1(S)$ through cleavage of the glucose moiety at the C20 position first and then glucose moiety at the C6 position (Fig. 3g and i in Fig. 4). For the PPT-type ginsenosides, BglAm also exhibited specific affinity to the glucose at positions C6 and C20, but not to rhamnoside at position C20. Eventually, BglAm exhibited a variety of ginsenoside [Rb_2, Rc, Rd, $Rg_3(S)$, Re, and Rg_1] hydrolyzing activities as follows: $Rb_2 \rightarrow$C-O$\rightarrow$C-Y, Rc$\rightarrow$C-$Mc_1\rightarrow$C-Mc, Rd$\rightarrow$ $F_2\rightarrow Rh_2(S)\rightarrow$PPD, $Rg_3(S)\rightarrow Rh_2(S)\rightarrow$PPD, Re$\rightarrow Rg_2(S)$, and $Rg_1\rightarrow Rh_1(S)\rightarrow$PPT, respectively (Fig. 5).

Discussion

The DNA sequence of BglAm was from the whole-genome sequenced *A. mirum* 101^T (Land et al. 2009). To the best our knowledge, BglAm has not yet been characterized before this research. BglAm is homologous to the glycoside hydrolase family 3 based on its amino acid sequence similarities to the β-glucosidase in *Arthrobacter* sp. FB24 (71.1 %, GenBank accession no. YP_832627), *Microbacterium testaceum* StLB037 (70.4 %, YP_004225291), and *T. ginsenosidimutans* Gsoil 3082^T (68.3 %, ACZ66247). The last sequence characterized by An et al. (2010) enabled the location of the BglAm function and led us to pursue ginsenoside biotransformation ability. β-Glucosidases have been primarily characterized in glycosyl hydrolase families 1 and 3, which commonly form in a closely related subfamily with a wide range of activities. Some β-glucosidases in this subfamily might not have only one specific activity, and they might have a 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 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) could be hydrolyzed.

The cloned ginsenoside-hydrolyzing GHs are in glycoside hydrolase families 1, 3, and 51, as listed in Table 2. Family 1 GHs convert Rb_1 to Rd, except β-glucosidase from *Sphingomonas* sp. 2F2 which converted Rb_1 to Gyp XVII (Wang et al. 2011b). Two cloned family 51 GHs have the same pathway of Rc$\rightarrow$Rd. Compared with family 1 and family 51 GHs, the three β-glucosidases belonging to family 3 GHs have more various types of ginsenoside-hydrolyzing pathways. The characterized ginsenoside-hydrolyzing GHs in family 3 enable the transformation of major ginsenosides: $Rb_2\rightarrow$Rd (Lee et al. 2011), $Rb_1\rightarrow$Gyp XVII$\rightarrow$Gyp LXXV (An et al. 2010), and $Rb_1\rightarrow Rg_3$(S) (Quan et al. 2012a), as listed in Table 2. Thus, family 3 GHs have greater potential to make various types of minor ginsenosides form major ginsenosides for application.

As a family 3 GH, BglAm exhibited a similar ginsenoside-transforming pathway to that of β-glucosidase from *T. ginsenosidimutans* Gsoil 3082^T (An et al. 2010); however, BglAm exhibits a $Rb_1\rightarrow Rh_2$ transformation pathway that has not been reported previously. Various minor ginsenosides [F_2, C-Mc_1, C-Mc, C-O, C-Y, $Rh_2(S)$, Gyp XVII, Gyp LXXV, PPD, $Rg_2(S)$, $Rh_1(S)$, and PPT] can be achieved from the major gisnensides (Rb_1, Rb_2, Rc, Rd, Rg_3, Re, and Rg_1) through deglycosylation by BglAm. BglAm also has various pathways in ginsenoside transformation compared to other cloned GHs (Table 2). This can be applied to produce various types of minor ginsenosides which may have more pharmacological activities. In further research, it will be meaningful to analyze the protein structure of family 3 GHs in order to uncover the mechanism of various ginsenoside-hydrolyzing pathways; BglAm could be a very important source.

In conclusion, a recombinant β-glucosidase (BglAm) belonging to glycoside hydrolase family 3 was constructed from *A. mirum* KACC 20028^T for use in the biotransformation of major ginsenosides. This enzyme was expressed in *E. coli* BL21(DE3) in a soluble form and was characterized; it had optimum reaction conditions at 37 °C and pH 7.0. BglAm could convert the major ginsenosides (Rb_1, Rb_2, Rc, Rd, Re, and Rg_1) into minor ginsenosides [F_2, C-Mc_1, C-Mc, C-O, C-Y, $Rh_2(S)$, PPD, Gyp LXXV, $Rg_2(S)$, $Rh_1(S)$, and PPT] through the selective hydrolysis of only the glucose moieties. This is the first report of the cloning of an enzyme that produces ginsenoside $Rh_2(S)$ through the biotransformation of ginsenosides Rb_1 via Gyp XVII and finally produces PPD.

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