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Enzymatic biotransformation of ginsenoside Rb1 to 20(*S*)-Rg3 by recombinant β-glucosidase from *Microbacterium esteraromaticum*

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Abstract *Microbacterium esteraromaticum* was isolated from ginseng field. The β-glucosidase gene (*bgp1*) from *M. esteraromaticum* was cloned and expressed in *Escherichia coli* BL21 (DE3). The *bgp1* gene consists of 2,496 bp encoding 831 amino acids which have homology to the glycosyl hydrolase family 3 protein domain. The recombinant β-glucosidase enzyme (Bgp1) was purified and characterized. The molecular mass of purified Bgp1 was 87.5 kDa, as determined by SDS-PAGE. Using 0.1 mg ml^{-1} enzyme in 20 mM sodium phosphate buffer at 37°C and pH 7.0, 1.0 mg ml^{-1} ginsenoside Rb1 was transformed into 0.444 mg ml^{-1} ginsenoside Rg3 within 6 h. The Bgp1 sequentially hydrolyzed the outer and inner glucose attached to the C-20 position of ginsenosides Rb1. Bgp1 hydrolyzed the ginsenoside Rb1 along the following pathway: Rb1→Rd→20(*S*)-Rg3. This is the first report of the biotransformation of ginsenoside Rb1 to ginsenoside 20 (*S*)-Rg3 using the recombinant β-glucosidase.

Keywords Biotransformation · β-glucosidase · Ginsenoside Rb1 · Ginsenoside 20(*S*)-Rg3

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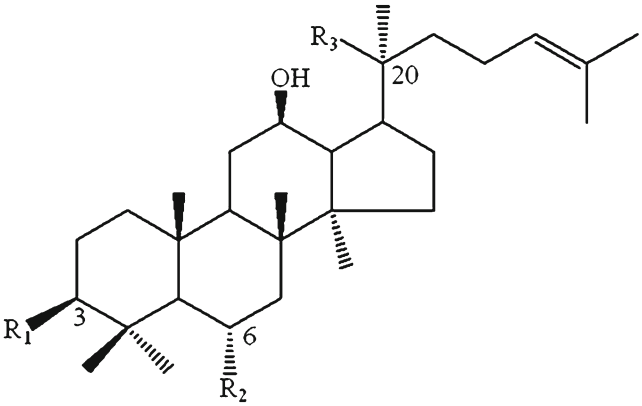
Introduction

Ginseng, the root of *Panax ginseng* C. A. Meyer, has been used as a traditional medicine in China, Korea, Japan, and other Asian countries (Ligor et al. 2005). As the major components in ginseng, ginsenosides have been reported to have various biological activities such as anti-tumor (Liu et al. 2000), anti-inflammatory (Kim et al. 1999), immune-modulatory (Lee et al. 1997), and anti-aging effects (Cho et al. 2006).

More than 40 ginsenosides have been isolated from ginseng roots (Cheng et al. 2007). They are classified into three groups: the oleanane, protopanaxadiol (PPD), and protopanaxatriol (PPT) types. The PPD ginsenosides have different sugar moieties at positions C-3 and C-20 in the aglycon PPD (Fig. 1). At position C-3, the PPD ginsenosides can have β-D-glucopyranose or β-D-glucopyranosyl-(1→2)-β-D-glucopyranose, position C-20 can have β-D-glucopyranose, α-L-arabinopyranosyl-(1→6)-β-D-glucopyranose, or α-L-arabinofuranosyl-(1→6)-β-D-glucopyranose. Ginsenoside Rg3, with a triterpenoid of dammarane skeleton as on aglycon and two glucose molecule moiety at C-3, has strong anti-metastatic (Mochizuki et al. 1995), tumor-suppressing (Shinkai et al. 1996), hepatoprotective (Lee et al. 2005), neuroprotective (Tian et al. 2005), and vasodilating effects (Kim et al. 2003). Because of the low Rg3 content in wild ginseng (i.e., less than 0.003%), Rg3 from natural products is quite expensive. Ginsenoside Rb1 which is abundant in ginseng (about 1–3%) is much easier to acquire and has a structure similar to Rg3. Enzymatically hydrolyzing two glucose molecules at C-20 can easily be transformed the ginsenoside Rb1 into Rg3.

Ginsenoside hydrolyzing enzymes can be purified directly or expressed recombinantly. Although purified enzymes have relatively high specificity, they are poorly expressed, and the



Ginsenoside (PPD)	R_1(C-3)	R_2(C-20)
Rb1	Glc(2→1)Glc	Glc(6→1)Glc
Rb2	Glc(2→1)Glc	Glc(6→1)Arap
Rc	Glc(2→1)Glc	Glc(6→1)Araf
Rd	Glc(2→1)Glc	Glc
Compound O	Glc	Glc(6→1)Arap
Compound Y	H	Glc(6→1)Arap
Compound Mc-1	Glc	Glc(6→1)Araf
Compound Mc	H	Glc(6→1)Araf
F2	Glc	Glc
Rg3	Glc(2→1)Glc	H
Rh2	Glc	H
Compound K	H	Glc

Fig. 1 Chemical structures of PPD ginsenosides. The ginsenosides represented here are all (*S*)-type ginsenosides. *Glc* β-D-glucopyranosyl, *Arap* α-L-arabinopyranosyl and *Araf* α-L-arabinofuranosyl

purification is a complex process with a low yield (0.1–12%). Recently, recombinant β-D-glycosidases from thermophilic organisms have been used to transform ginsenosides with high expression and high purification yields (Kim et al. 2007; Noh and Oh. 2009; Noh et al. 2009). Purification can be conducted in one step, such as heat treatment or His-Trap affinity chromatography. Moreover, the recombinant enzymes generate minor ginsenosides at relatively high yields because they can hydrolyze several ginsenosides simultaneously.

This study is the first report about the cloning and expression of Bgp1, the ginsenoside-hydrolyzing β-glucosidase from *Microbacterium esteraromaticum*. The enzyme hydrolyzed two glucose moieties attached to the C-20 position of Rb1 effectively converting it into Rd and Rg3.

Materials and methods

Materials

Ginsenosides Rb1, Rc, Rd, F2, Rg3, Rh2, and compound K were obtained from Ginseng Genetic Resource Bank (Kyung-Hee University, Yongin, Korea). 5-Bromo-4-chloro-3-indolyl β-D-glucopyranoside (*X*-Glu), *p*-nitrophenyl (*p*NP)-β-D-glucopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-α-L-arabinopyranoside, *o*-nitrophenyl (*o*NP)-β-D-glucopyranoside, and *o*NP-β-D-galactopyranoside were purchased from Sigma.

Molecular cloning, expression, and purification of recombinant Bgp1

M. esteraromaticum (KCTC 12040BP, Korean Collection for Type Cultures, Daejeon, Korea) was isolated from ginseng field. The genomic DNA from *M. esteraromaticum* was extracted using the genomic DNA extraction kit. The gene-encoded β-glucosidase was amplified from the genomic DNA as a template by polymerase chain reaction (PCR) using *Pfu* DNA polymerase. The gene termed *bgp1* (GenBank accession number JN 603820) was amplified using the following primers (with *Nde*I and *Eco*RI restriction sites in boldface): *bgp1*F (5′-GGC C**CA TAT G**TG CGG ATG CCC TAC C-3′) and *bgp1*R (5′-G**GA ATT C**CT ATC GGG CCG C-3′). The amplified fragment was digested with *Nde*I and *Eco*RI and inserted into pMAL-c5X to generate a maltose-binding protein (MBP)-*bgp1* gene fusion. The amplified gene (*bgp1*) was sequenced and confirmed at the facility of Genotech (Daejeon, Korea).

Escherichia coli BL21 (DE3) transformed with recombinant pMAL-*bgp1* were grown in Luria–Bertani (LB)–

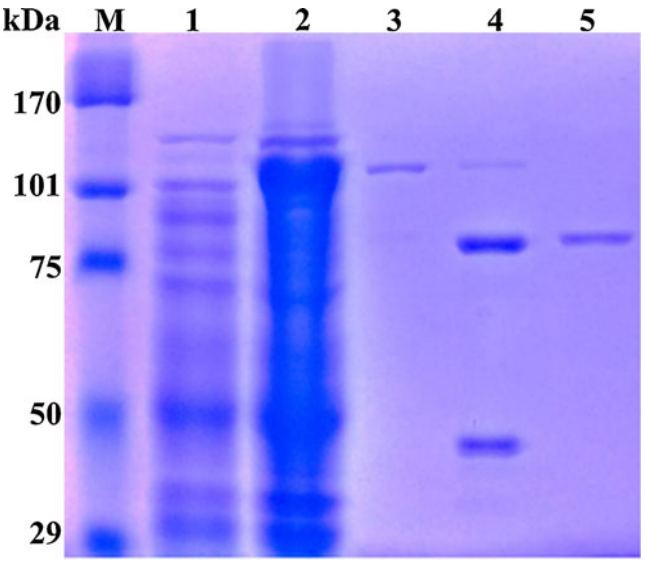


Fig. 2 Purification of recombinant Bgp1. *M* molecular mass markers; *lane 1* crude extract of uninduced BL21 (DE3) cells carrying pMAL-Bgp1; *lane 2* crude extract of induced recombinant BL21 (DE3) cells; *lane 3* amylose affinity purified pMAL-Bgp1; *lane 4* factor Xa digested fusion protein showing Bgp1 and MBP; *lane 5* purified Bgp1 protein eluted from the second amylose column

Table 1 Purification scheme for the recombinant protein (Bgp1)

Purification steps	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg)	Purification (fold)	Yield (%)
Cell-free extract	2,441	80.5	30.3	1	100
First amylose affinity chromatography	2,020	36	56	1.8	82.7
Second amylose affinity chromatography	1,573.2	25.2	61	2.0	64.4

[a] One unit of Bgp1 was defined as the amount of enzyme liberating 1 μmol min^{-1} of *p*NP

ampicillin medium at 37°C until they reached an optical density at 600 nm (OD_{600}) of 0.4, at which point protein expression was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria were incubated for additional 9 h at 28°C and harvested by centrifuging at 5,000×*g* for 30 min at 4°C. The cells were washed twice with 20 mM sodium phosphate buffer (pH 7.0, 1 mM EDTA, and 1 mM NaCl) and then resuspended in 20 mM sodium phosphate buffer (pH 7.0). Cells were lysed by sonication with short pulses, and debris was removed by centrifugation (12,000×*g*, at 4°C for 30 min). The crude extract of 5 ml cell lysate was applied to the preequilibrated amylose column (5 ml, NEB, USA) followed by washing with 12 column volumes of elusion buffer (20 mM sodium phosphate buffer pH 7.0, 1 mM EDTA, and 1 mM NaCl). Bound proteins were eluted with elusion buffer supplemented with 10 mM maltose. The fractions containing the fusion protein were pooled and concentrated and followed by cleavage with protease factor Xa in cleavage buffer (20 mM sodium phosphate buffer pH 7.0, 1 mM EDTA, 1 mM NaCl, and 10 mM maltose) to separate the MBP and Bgp1. The cleaved reaction products were applied to the second amylose column, and Bgp1 was purified and collected in the flow-through. The protein homogeneity was assessed by 10% SDS-PAGE followed by Coomassie blue staining.

Enzyme characterization

The specific activity of purified Bgp1 was determined using *p*NP-β-D-glucopyranoside as a surrogate substrate in 20 mM sodium phosphate buffer, pH 7.0 at 37°C. Reactions were stopped after 5 min by adding Na_2CO_3 at a final concentration of 0.1 M, and *p*NP release was measured immediately using a microplate reader at 405 nm. One unit of activity was defined as the amount of protein required to generate 1 μmol of *p*NP per min. Specific activity was expressed as units per milligram of protein. Protein concentration was determined using the SMART™BCA Protein Assay Kit (Intron, Sengnam, Korea).

To determine optimal pH for the Bgp1, *p*NP-β-D-glucopyranoside hydrolyzing activity was studied at 37°C in the buffers at various pH (3.0–9.0). To determine the optimal temperature for the Bgp1, β-glucosidase activity was studied at 20–70°C in 20 mM sodium phosphate buffer (pH 7.0).

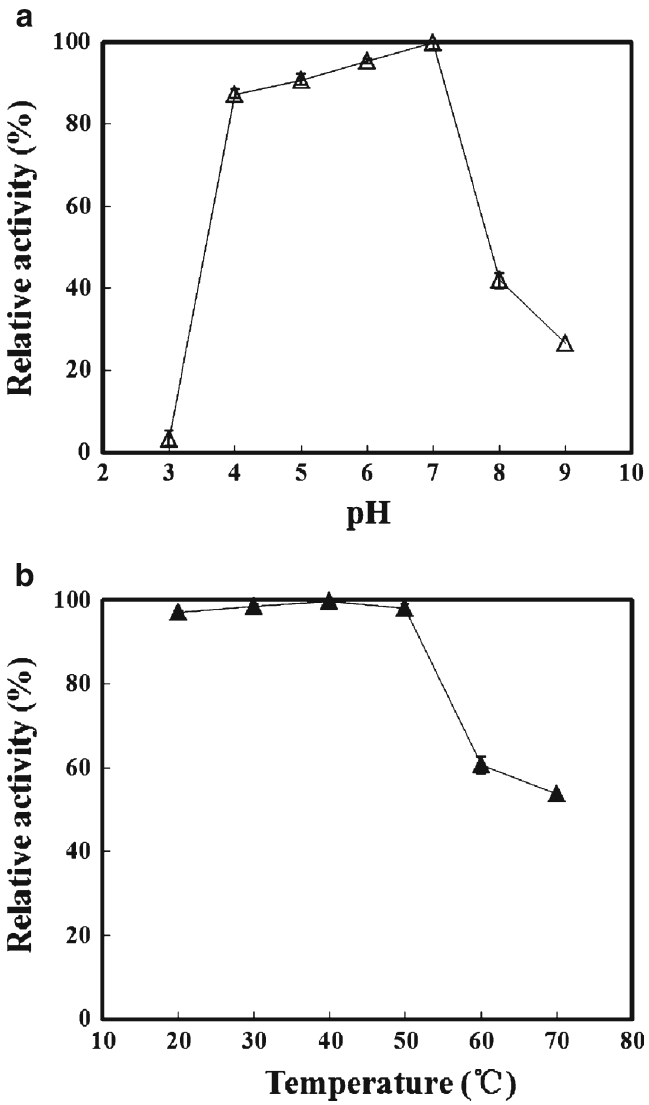


Fig. 3 Effect of pH (**a**) and temperature (**b**) on the activity of recombinant Bgp1 determined using *p*NP-β-D-glucopyranoside as a substrate. The following buffers (20 mM) were tested: glycine–HCl buffer (pH 3.0), citric acid-sodium citrate buffer (pH 4.0–5.0), sodium phosphate buffer (pH 6.0–7.0), Tris–HCl buffer (pH 8.0–9.0). Effect of temperature on enzyme activity was tested in 20 mM sodium phosphate buffer pH 7.0

Table 2 Relative activity of purified recombinant Bgp1 toward various chromogenic substrates as measured by *o*NP or *p*NP release at 37°C

Substrate[a]	Relative activity±SD (%)[b]
*p*NP-α-D-glucopyranoside	0
*p*NP-α-L-arabinofuranoside	0
*p*NP-β-D-galctopyranoside	0
*p*NP-α-L-arabinopyranoside	0
*p*NP-β-D-glucopyranoside	100±1.1
*o*NP-β-D-glucopyranoside	13.4±0.9
*o*NP-β-D-galactopyranoside	6.7±0.8

[a] Final concentration, 2.0 mM

[b] The activity against *p*NP-β-D-glucopyranoside was assumed to be 100%

Substrate preference was examined using 2.0 mM chromogenic *o*NP and *p*NP substrates at 37°C for 5 min with one unit of enzyme activity was defined as the releases 1 μmol *o*NP or *p*NP per minute. The following substrates were tested, *p*NP-β-D-glucopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-α-L-arabinopyranoside, *o*NP-β-D-glucopyranoside, and *o*NP-β-D-galactopyranoside.

Enzymatic hydrolysis of major ginsenosides

Initial biotransformation experiments using ginsenoside Rb1 as a substrate revealed that the presence of MBP fused to Bgp1 did not affect the enzyme activity. Therefore, the MBP fusion protein was used to determine the specificity and selectivity of Bgp1 for the hydrolysis of glucose moieties attached at the C-3 and C-20 positions of ginsenosidses Rb1, Rc, Rd, and F2. Bgp1 (0.1 mg/ml) in sodium phosphate buffer (pH 7.0) was incubated with an equal volume of 1 mg/ml Rb1, Rc, Rd, or F2 in 20 mM sodium phosphate buffer (pH 7.0) at 37°C. Samples were withdrawn at regular intervals. An equal volume of water-saturated *n*-butanol was added to each sample to stop the reaction; subsequently, the *n*-butanol fraction was evaporated to dryness, and the methanol extract was analyzed by TLC, HPLC, and LC/MS.

TLC analysis of ginsenosides

TLC was performed with silica gel plates (60 F_{254}, Merck, Darmstadt, Germany), with the developing solvent $CHCl_3$:CH_3OH:H_2O (65:35:10, *v*/*v*, lower phase). Spots on the TLC plates were detected by spraying plates with 10% H_2SO_4 followed by heating at 110°C for 10 min.

HPLC and LC/MS analysis for biotransformation of ginsenosides

The HPLC-grade acetonitrile and water were purchased from SK Chemicals (Ulsan, Korea). The reaction mixture was extracted in *n*-butanol saturated with H_2O and evaporated in a vacuum. The residue was dissolved in CH_3OH for HPLC analysis. This experiment employed a C_{18} (250×4.6 mm, particle size 5 μm) column using acetonitrile (solvent A) and distilled water (solvent B) mobile phases at 85% B for 5 min, 79% B for 20 min, 42% B for 55 min, 10% B for 12 min, and 85% B for 18 min at a flow rate of 1.6 ml min^{-1}. The sample was detected by UV 203 nm. LC/MS for ginsenosides was analyzed by Agilent QQQ/MS with positive polarity and an ion trap analyzer. Ion spray was operated under 5 l/min N_2, 3.5 kV, 25 psi, and 300°C.

Results

Expression and purification of recombinant Bgp1

A gene consists of 2,496 bp encoding 831 amino acids which have homology to the glycosyl hydrolase families 3 protein domain (Varghese et al. 1999), with the same sequence as that *bgp1* (GenBank accession number JN 603820), was cloned and expressed in *E. coli.*

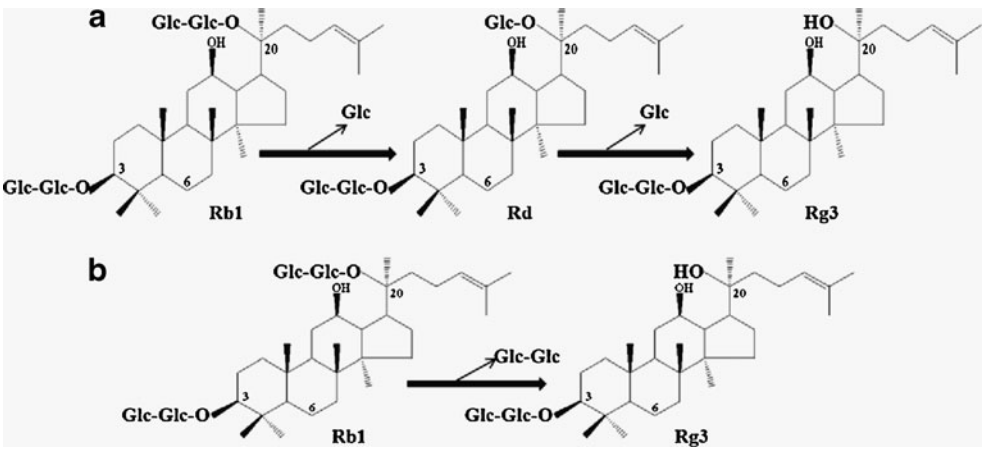


Fig. 4 Transformation pathways from ginsenoside Rb1 into Rg3 by Bgp1

To maximize yield of the fusion protein were tested with different condition for protein induction. Induction with 0.5 mM IPTG at 28°C for 9 h produced the maximum level of soluble active fusion enzyme. The MBP-Bgp1 fusion protein was purified from cell-free bacterial lysate by affinity chromatography on amylose resin. Most of the fusion protein eluted within the first few fractions after adding maltose to the elusion buffer. SDS-PAGE of the eluted protein fractions showed a single band of 130 kDa (Fig. 2). After digesting the purified fusion protein with factor Xa protease, two protein bands appeared, which migrated with apparent molecular masses of 87.5 and 42.5 kDa by SDS-PAGE, corresponding to Bgp1 and MBP, respectively (Fig. 2). These digested proteins were again purified by chromatography on second amylose column, which bound the cleaved MBP tag, while unbound pure Bgp1 was collected in the flow-through. The molecular weight of the purified Bgp1 was 87.5 kDa as determined by SDS-PAGE (Fig. 2). This procedure resulted in 2.0-fold purification and 64.4% recovery from the crude extract (Table 1).

Enzyme characterization

At 37°C, the enzyme showed optimal activity at pH 7.0 in 20 mM sodium phosphate buffers (Fig. 3a). The optimal temperature for Bgp1 activity was 40°C (Fig. 3b).

The substrate specificity of Bgp1 was tested using 2.0 mM *p*NP and *o*NP glycosides with α and β configurations (Table 2). The hydrolytic activity for the *p*NP and *o*NP substrates was as follows: *p*NP-β-D-glucopyranoside>*o*NP-β-D-glucopyranoside>*o*NP-β-D-galactopyranoside. The enzyme exhibited no activity toward *p*NP-α-D-glucopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-α-L-arabinopyranoside, or *p*NP-β-D-galactopyranoside.

Biotransformation pathway of ginsenoside Rb1 by Bgp1

Theoretically, there are two pathways for conversion of ginsenoside Rb1 to Rg3 (Fig. 4). One is through the ginsenoside Rd by sequential hydrolyzed of two glucose attached to the C-20 position of ginsenoside Rb1 (Fig. 4a). The other is direct hydrolysis of the inner glucose at C-20 (Fig. 4b). To order to investigate the pathway by which a ginsenoside transforms Rb1 into Rg3, ginsenosides Rb1, Rc, Rd, and F2 were used as substrates. As shown in Fig. 5a, Bgp1 completely converted ginsenoside Rd into Rg3 within 4 min, while it converted the ginsenoside Rb1 into Rg3 within 6 h (Fig. 5b). These results demonstrated that the enzyme converted Rd into Rg3 faster than Rb1 into Rg3.

To investigate whether Bgp1 showed the same specificity and selectivity for glycone residues attached at the C-3 and

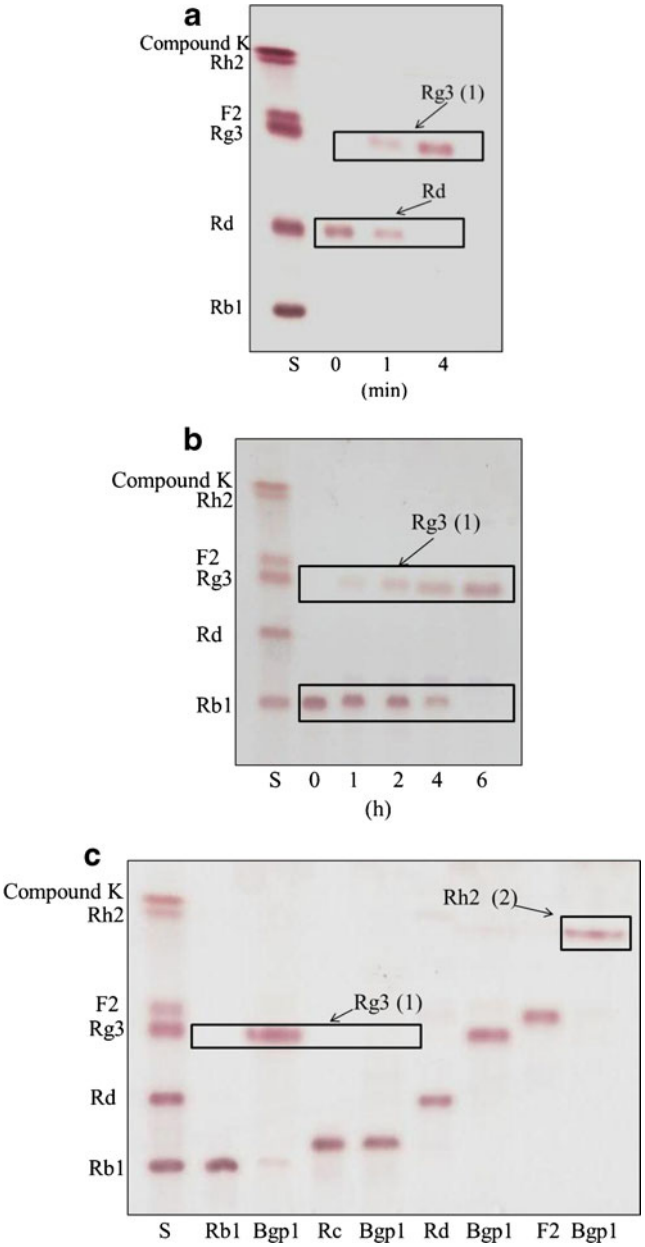


Fig. 5 TLC analysis of ginsenosides transformation by Bgp1. **a** Time-course of transformation of ginsenoside Rd by the Bgp1. **b** Time-course of transformation of ginsenoside Rb1 by the Bgp1. **c** Transformation of ginsenoside Rb1, Rc, Rd, and F2 by the Bgp1. *S* standard; *Rg3 (1)* metabolites 1; *Rh2 (2)* metabolites 2

C-20 positions of other PPD ginsenosides, the ginsenosides Rc and F2 were also used as substrates. Ginsenoside Rc has one arabinofuranose and one glucose attached at the C-20 position and two glucose attached at the C-3 position, while F2 has only one glucose attached at both the C-20 and C-3 positions (Fig. 1). Ginsenoside F2 was converted to Rh2 by cleavage of the glucose at the C-20 position (Fig. 5c). However, when ginsenoside Rc was used as substrate, Bgp1 did not hydrolyze outer arabinofuranose and inner glucose moiety at the C-20 and two glucose moiety at the C-3 position of

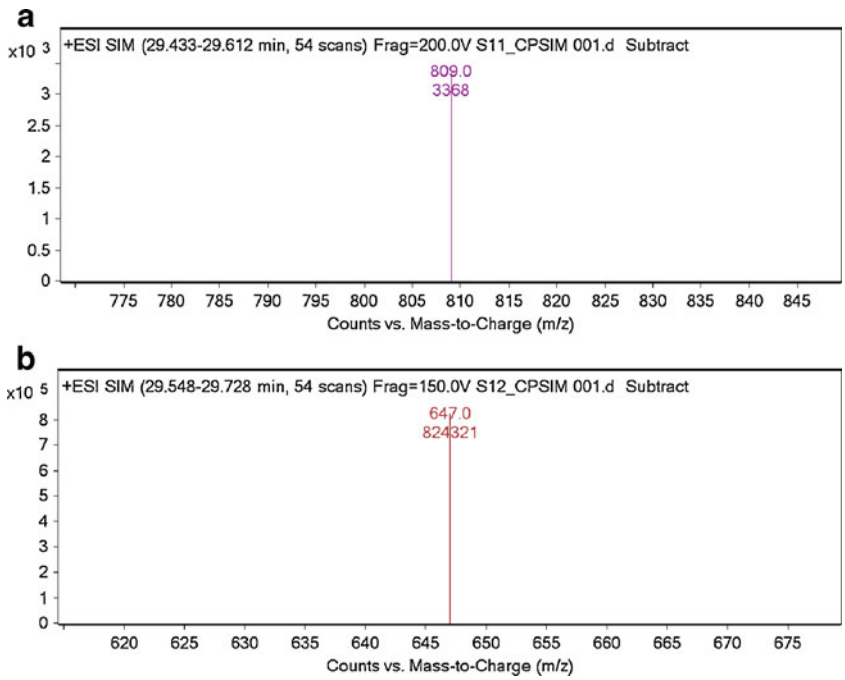


Fig. 6 Mass spectra of ginsenosides Rb1 and F2 after hydrolysis by Bgp1. **a** Mass spectrum of Rg3, *m/z* 809=[Mw+Na+H], Mw 785. **b** Mass spectrum of Rh2, *m/z* 647=[Mw+Na+H], Mw=623

ginsenoside Rc (Fig. 5c). Hence, we concluded that the hydrolysis pathway of ginsenoside Rb1 by Bgp1 was Rb1→Rd→20(*S*)-Rg3 by stepwise hydrolysis of the outer and inner glucose attached at the C-20 position of Rb1 (Fig. 4a).

Metabolites 1 and 2 from the transformation were subjected to LC/MS to determine their molecular weights. Metabolite 1 was a white powder with a pseudomolecular ion peak [M+Na+H] at *m/z* 809 in ESIMS, corresponding to $C_{42}H_{72}O_{13}$ (calculated molecular weight, 785). Thus, metabolite 1 was confirmed as ginsenoside Rg3 (Fig. 6a).

The molecular formula of metabolite 2 was $C_{36}H_{62}O_8$ based on the protonated molecular ion peak [M+Na+H] at *m/z* 647 (Mw 623) in ESIMS. Thus, metabolite 2 was confirmed as ginsenoside Rh2 (Fig. 6b).

The conversion yield of ginsenoside Rb1 by Bgp1 was confirmed quantitatively by HPLC analysis. As shown in Fig. 7, 1.0 mg ml^{-1} ginsenoside Rb1 was transformed into 0.201 mg ml^{-1} ginsenoside Rg3 at 2 h. After 6 h, almost all ginsenoside Rb1 was transformed to 0.444 mg ml^{-1} ginsenoside Rg3 (1). Thus, Bgp1 transformed 1.0 mg ml^{-1} ginsenoside Rb1 to 0.444 mg ml^{-1} ginsenoside Rg3 corresponding to a mole conversion yield of 71%.

Discussion

The β-glucosidase gene (*bgp1*) from *M. esteraromaticum* was cloned and expressed in *E. coli* BL21 (DE3). The recombinant enzyme (Bgp1) was purified and characterized. The molecular weight of the purified Bgp1 was 87.5 kDa, as determined by SDS-PAGE showing lower than previous reported ginsenoside-hydrolyzing β-glucosidase (102 kDa) isolated from *Paecilomyces Bainier* sp. 229 (Yan et al. 2008a). The enzyme had optimal activity at pH 7.0 higher than that of ginsenoside-hydrolyzing β-glucosidase (pH 3.5) isolated from *Paecilomyces Bainier* sp. 229 (Yan et al. 2008a). The optimal temperature for Bgp1 activity was 40°C which was lower than the optimal temperatures for ginsenoside-hydrolyzing β-glucosidases (55°C) from *Paecilomyces Bainier* sp. 229 and *Sulfolobus acidocaldarius* (85°C) (Yan et al. 2008a; Noh and Oh. 2009).

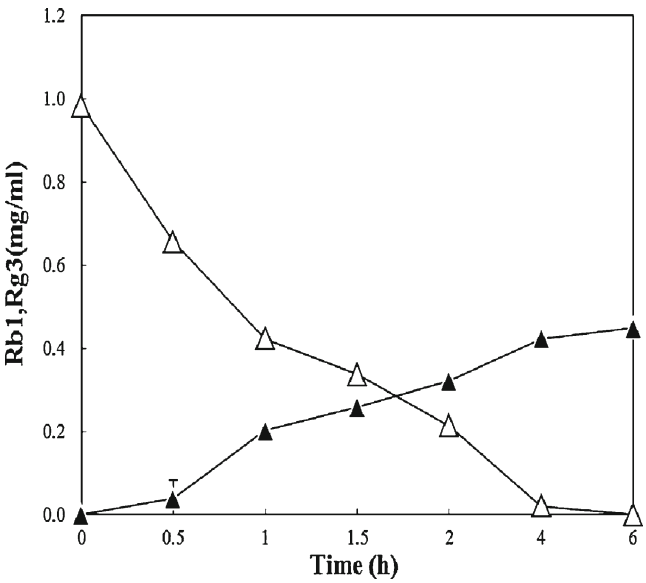


Fig. 7 Time course for ginsenoside Rg3 production from ginsenoside Rb1 by Bgp1 under the optimum conditions. Rb1 (*empty triangle*)→Rg3 (*filled triangle*)

Theoretically, four glucose moieties attached to the ginsenoside Rb1 could be available for hydrolysis by Bgp1, namely the outer and inner glucose moieties attached at positions C-3 and C-20. Based on an analysis of the hydrolysis products of ginsenoside Rb1, Bgp1 acted on not C-3 position but C-20 position, hydrolyzed two glucose moieties attached to the C-20 position of Rb1, effectively converted into Rg3. Theoretically, there are two pathways to hydrolyze ginsenoside Rb1 into Rg3. One is by sequential hydrolysis of the two glucoses at C-20 of Rb1 with ginsenoside Rd as an intermediate. The other pathway is directly hydrolyzing the inner glucose at C-20 of Rb1. The conversion of Rd into Rg3 by Bgp1 was faster than conversion of Rb1 into Rg3. Furthermore, when the ginsenoside Rc was a substrate, inner glucose attached to the C-20 positions of ginsenoside Rc was not directly hydrolyzed. These results indicated that Bgp1 showed substrate specificity for PPD ginsenosides that sequential hydrolyses of two glucoses attached at the C-20 but did not direct hydrolyses of the inner glucose at C-20. Hence, Bgp1 hydrolyzed the ginsenoside Rb1 along the pathway Rb1 → Rd → 20(*S*)-Rg3 and hydrolyze the outer and inner glucose molecules at position C-20 sequentially (Fig. 4a).

Ginsenoside Rg3 production has been achieved from ginsenosides Rb1, Rb2, Rc, and Rd using enzymatic methods, including use of the lactase from *Penicillium* sp. (Ko et al. 2007) and purified β-glucosidase from *Paecilomyces Bainier* sp. 229 (Yan et al. 2008a). Although purified enzymes usually have relatively high specificity, purification yields (0.1–12%), and cell expression are usually rather low and the purification process is complex (Andreea Neculai et al. 2009; Park et al. 2001; Yan et al. 2008a; Yan et al. 2008b; Yu et al. 2007). Through the several purification steps, the purified enzyme converting ginsenosides was not efficient to apply in industrial use. Recently, recombinant β-D-glycosidases from thermophilic microbes have been used for ginsenoside transformation (Kim et al. 2007; Son et al. 2008; Noh and Oh 2009; Noh et al. 2009; Yoo et al. 2011), but they mainly convert ginsenoside Rb1 to F2, compound K, and aglycon PPD. Conversion of ginsenoside Rg3 was not reported.

This study demonstrated that ginsenoside Rg3 was produced from the ginsenoside Rb1 via ginsenoside Rd by a recombinant β-glucosidase from *M. esteraromaticum*. The recombinant Bgp1 provides effective ginsenoside transformation with high productivity and low cost. Therefore, the Bgp1 enzyme is considered be potentially useful for the practical preparation of ginsenoside Rg3.

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