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# Enzymatic Biotransformation of Ginsenoside Rb1 to Compound K by Recombinant β-Glucosidase from *Microbacterium esteraromaticum*

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**ABSTRACT:** We cloned and characterized a β-glucosidase (*bgp3*) gene from *Microbacterium esteraromaticum* isolated from ginseng field. The *bgp3* gene consists of 2,271 bp encoding 756 amino acids which have homology to the glycosyl hydrolase family 3 protein domain. The molecular mass of purified Bgp3 was 80 kDa, as determined by SDS−PAGE. The enzyme (Bgp3) catalyzed the conversion of ginsenoside Rb1 to the more pharmacologically active minor ginsenoside Rd and compound K. The Bgp3 hydrolyzed the outer glucose moiety attached to the C-20 position of ginsenoside Rb1, followed by hydrolysis of the inner glucose moiety attached to the C-3 position. Using 0.1 mg $mL^{-1}$ enzyme in 20 mM sodium phosphate buffer at 40 °C and pH 7.0, 1.0 mg $mL^{-1}$ ginsenoside Rb1 was transformed into 0.46 mg $mL^{-1}$ compound K within 60 min with a corresponding molar conversion yield of 77%. Bgp3 hydrolyzed the ginsenoside Rb1 along the following pathway: Rb1 → Rd → compound K.

**KEYWORDS:** *biotransformation, β-glucosidase, ginsenoside Rb1, compound K*

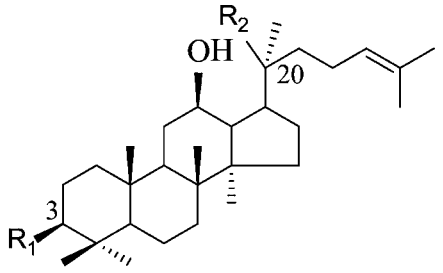
## 1. INTRODUCTION

*Panax ginseng* C. A. Mayer is used in traditional medicine to strengthen immunity, provide nutrition, and reduce fatigue. The major active ingredients of ginseng are triterpene glycosides known as ginsenosides.[1,2] Many previous studies have focused on the anticancer, anti-inflammatory, antiproliferative, and antitumor activities.[3−6]

More than 40 ginsenosides have been isolated from ginseng roots.[7] They are classified into three groups: oleanane, protopanaxadiol (PPD), and protopanaxatriol (PPT). The PPD ginsenosides harbor different sugar moieties at C-3 and C-20 in the aglycon PPD (Figure 1). The sugars linked to C-3 in the PPD ginsenosides are β-D-glucopyranose and β-D-glucopyranosyl-(1→2)-β-D-glucopyranose, whereas those linked to C-20 are β-D-glucopyranose, α-L-arabinopyranosyl-(1→6)-β-D-glucopyranose, and α-L-arabinofuranosyl-(1→6)-β-D-glucopyranose. The minor ginsenosides, including ginsenosides F2, Rg3, Rh2, compound K, Rg2, F1, and Rh1, can be produced by hydrolysis of sugar moieties from the major ginsenosides Rb1, Rb2, Rc, Rd, Re and Rg1, which comprise more than 80% of the total ginsenosides.[8]

The deglycosylated compound K, which is absent in ginseng root, reportedly induces tumor cell apoptosis, inhibits tumor metastasis, and restrains tumor invasion.[9−11] Its production has been achieved from ginsenosides Rb1, Rb2, Rb3, and Rd using enzymatic methods including use of the crude enzyme from *Leuconostoc citreum* LH1,[12] crude enzyme from *Lactobacillus pentosus* DC101,[13] crude enzyme from *Leuconostoc mesenteroides* DC102,[14] β-D-glucosidase from *Fusobacterium* sp,[15] β-D-galactosidase from *Aspergillus oryzae*,[16] Lactase from *Penicillium* sp.[16] and β-D-glucosidase from *Aspergillus* sp.[17] However, these enzymes exhibited low selectivity and poor productivity.

In this study, we report about the cloning and expression of Bgp3, the ginsenoside-hydrolyzing β-glucosidase from *Microbacterium esteraromaticum*. The enzyme hydrolyzed the outer glucose moiety attached to the C-20 position, followed by hydrolysis of the inner glucose moiety attached to the C-3 position effectively converting it into compound K.




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

**Figure 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.

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## 2. MATERIALS AND METHODS

**2.1. 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.

**2.2. TLC Analysis of Ginsenosides.** TLC was performed with silica gel plates ($60F_{254}$, 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.

**2.3. Analysis of Ginsenosides by HPLC and LC/MS.** The reaction mixture was extracted with *n*-butanol saturated with $H_2O$, and evaporated in a vacuum. The residue was dissolved in methanol and analyzed by HPLC using a $C_{18}$ (250 × 4.6 mm, particle size 5 μm) column with acetonitrile (solvent A) and distilled water (solvent B) as 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 all at 1.6 mL $min^{-1}$. Detection was at 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 $N_2$/min, 3.5 kV, 25 psi, and 300 °C.

**2.4. Molecular Cloning, Expression, and Purification of Recombinant Bgp3.** *Microbacterium esteraromaticum* (KACC 16318, Korean Agricultural Culture Collection, Suwon, Korea) was isolated from a ginseng field. Genomic DNA from *Microbacterium esteraromaticum* was extracted using the genomic DNA extraction kit. The gene-encoded β-glucosidase was amplified from the genomic DNA and used as a template by polymerase chain reaction with *Pfu* DNA polymerase. The gene, termed *bgp3* (GenBank accession number JN 603821), was amplified using the following primers (with *Nde*1 and *Eco*RV restriction sites in boldface): *bgp3*F (5′-C**CA TAT G**GA GCC CCA GAT GAC CAA-3′) and *bgp3*R (5′-CC**G ATA TC**T CAG GCG AAG ACG-3′). The amplified fragment was digested with *Nde*1 and *Eco*RV and inserted into pMAL-c5X to generate a maltose-binding protein (MBP)-*bgp3* gene fusion. The amplified gene (*bgp3*) was sequenced and confirmed.

*Escherichia coli* BL21 (DE3), transformed with recombinant pMAL-*bgp3*, was grown in Luria−Bertani (LB)−ampicillin medium at 37 °C to an optical density at 600 nm ($OD_{600}$) of 0.4; 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 5000*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 sonicated with short pulses, and debris was removed by centrifugation (12000*g*, at 4 °C for 30 min). The crude extract of 5 mL of cell lysate was applied to the pre-equilibrated amylose column, followed by washing with 12 column volumes of elution buffer (20 mM sodium phosphate buffer pH 7.0, 1 mM EDTA, and 1 mM NaCl). The bound proteins were eluted with elution buffer supplemented with 10 mM maltose. The fractions containing fusion protein were pooled and concentrated and followed by cleavage with protease factor Xa in the buffer (20 mM sodium phosphate buffer pH 7.0, 1 mM EDTA, 1 mM NaCl, and 10 mM maltose), and finally the fusion protein was separated to the MBP and Bgp3. The cleaved proteins were applied to the second amylose column, and Bgp3 was eluted with flow-through and purified. The protein homogeneity was assessed by 10% SDS−PAGE followed by Coomassie blue staining.

**2.5. Enzyme Characterization.** The specific activity of purified Bgp3 was determined using *p*NP-β-D-glucopyranoside as substrate in 20 mM sodium phosphate buffer, pH 7.0, at 40 °C. Reactions were stopped after 5 min by adding $Na_2CO_3$ to give 50 mM. The released *p*NP 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.

To determine optimal pH for the Bgp3, *p*NP-β-D-glucopyranoside hydrolyzing activity was studied at 40 °C in buffer at various pH (3.0−9.0). The pH stability of the enzyme was determined by measuring remaining enzymatic activity after incubation in each buffer (pH 3.0−9.0) for 4 h at 40 °C. The effect of temperature on enzymatic activity was tested after incubation of the enzyme at various temperatures ranging from 20 to 70 °C for 5 min in optimum pH in 20 mM sodium phosphate buffer containing 10 mM *p*NP-β-D-glucopyranoside. The thermostability of the enzyme was examined after 4 h incubation at pH 7.0 in different temperature.

The effects of metals and other chemicals on Bgp3 activity were determined. Bgp3 activity was tested in the presence of 10 mM (final concentration) NaCl, KCl, $CaCO_3$, $MnSO_4$, $FeSO_4$, $MgSO_4$, and SDS for 5 min at 40 °C. The remaining activity was determined using *p*NP-β-D-glucopyranoside as a substrate, and activities are expressed as a percentage of the activity obtained in the absence of compound.

Substrate preference was examined using 10 mM chromogenic *o*NP and *p*NP substrates at 40 °C for 5 min with one unit of enzyme activity defined as the release of 1 μmol of *o*NP or *p*NP per min. 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.

**2.6. Enzymatic Hydrolysis of Ginsenosides.** Initial biotransformation experiments using ginsenoside Rb1 as a substrate showed that the presence of MBP fused to Bgp3 did not affect the enzyme activity. Therefore, the fused enzyme at 0.1 mg $mL^{-1}$ dissolved in 20 mM sodium phosphate buffer (pH 7.0) was incubated with an equal volume of ginsenosides Rb1, Rc, Rd, and F2 at 1 mg $mL^{-1}$ in 20 mM sodium phosphate buffer (pH 7.0) at 40 °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, *n*-butanol fraction was evaporated to dryness, and the methanol extract was analyzed by TLC, HPLC, and LC/MS.

## 3. RESULTS AND DISCUSSION

**3.1. Expression and Purification of Recombinant Bgp3.** A gene consists of 2,271 bp encoding 756 amino acids, which have homology to the protein domain of glycosyl hydrolase families 3[18] and with the same sequence as of the *bgp3* (GenBank accession number JN 603821), was cloned and expressed in *E. coli*.

The MBP-Bgp3 fusion protein was purified from cell-free lysate by affinity chromatography using an amylose resin. Most of the fusion protein eluted within the first few fractions after addition of maltose to the elution buffer. SDS−PAGE of the eluted protein fractions showed a single band of 122.5 kDa (Figure 2). After digestion of the purified fusion protein with factor Xa protease, two protein bands appeared, which migrated with apparent molecular weight ($M_w$) of 80.0 and 42.5 kDa by SDS−PAGE, corresponding to Bgp3 and MBP, respectively (Figure 2). These digested proteins were again purified by chromatography on a second amylose column, which bound the cleaved MBP tag, while unbound pure Bgp3 was collected in the flow-through. The $M_w$ of the purified Bgp3 was 80 kDa as determined by SDS−PAGE (Figure 2). A β-glycosidase from *Pyrococcus furious*,[19] β-D-glucosidase from *Paecilomyces bainier*,[20] and β-glucosidase from *Terrabacter ginsenosidimutans* sp.[21] were 55.5, 115, and 70 kDa, respectively. This procedure resulted in 1.8-fold purification and 11.5% recovery from the crude extract (Table 1).

**3.2. Enzyme Characterization.** The optimum pH was pH 7.0 in 20 mM sodium phosphate buffer (Figure 3). A β-glycosidase from *Pyrococcus furious*,[19] β-D-glucosidase from

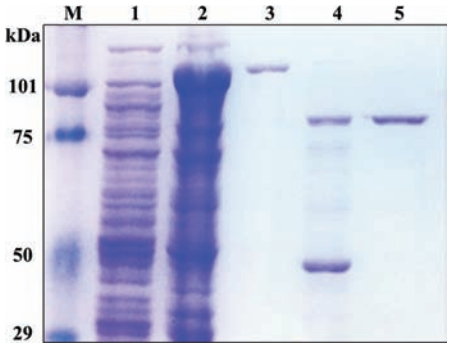


**Figure 2.** Purification of recombinant Bgp3: M, molecular mass markers; lane 1, crude extract of uninduced BL21 (DE3) cells carrying pMAL-Bgp3; lane 2, crude extract of induced recombinant BL21 (DE3) cells; lane 3, amylose affinity purified pMAL-Bgp3; lane 4, factor Xa digested fusion protein showing Bgp3 and MBP; lane 5, purified Bgp3 protein eluted from the second amylose column.

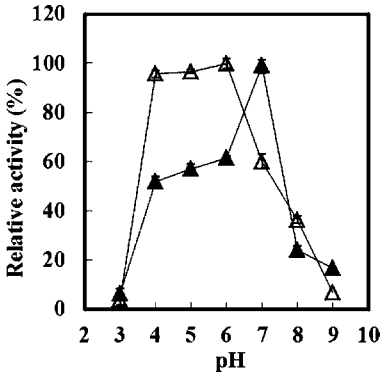


**Figure 3.** Effect of pH on the stability and the activity of recombinant Bgp3 determined using *p*NP-*β*-D-glucopyranoside as a substrate. The following buffers (20 mM) were tested: glycine/HCl buffer (pH 3.0), citric acid/sodium citrate buffer (pH 4.0−5.0), sodium phosphate buffer (pH 6.0−7.0), and Tris/HCl buffer (pH 8.0−9.0). ▲, enzyme activity. Maximum activity observed at pH 7.0 was taken as 100% = 29.4 U (mg protein)$^{-1}$. Δ, stability. Enzyme activity was assayed under standard conditions (pH 7.0, 40 °C) after incubation at 40 °C for 4 h at different pH levels. The original activity before incubation was taken as 100%.

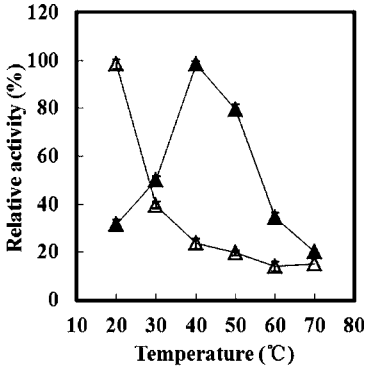


**Figure 4.** Effect of temperature on the stability and activity of recombinant Bgp3 determined using *p*NP-*β*-D-glucopyranoside as a substrate. ▲, the thermodependence of enzyme activity was assayed in 20 mM sodium phosphate buffer (pH 7.0) at various temperatures ranging from 20 to 70 °C. Maximum activity observed at 40 °C was taken as 100% = 29.4 U (mg protein)$^{-1}$. Δ, thermostability was tested by incubating aliquots of the enzyme in 20 mM sodium phosphate buffer (pH 7.0) for 4 h at different temperatures.

*Paecilomyces bainier*,[20] and *β*-glucosidase from *Terrabacter ginsenosidimutans* sp.[21] had optima at pH 3.5, 5.5 and 7.0, respectively. The effect of pH on enzyme stability is shown in Figure 3. After incubation from pH 4.0−6.0 at 40 °C for 4 h, the enzyme still retained more than 80% of its original activity.

The optimal temperature for Bgp3 activity was 40 °C (Figure 4), which was lower than the ginsenoside hydrolyzing *β*-glycosidase (95 °C) from *Pyrococcus furious*[19] and *β*-D-glucosidase (55 °C) from *Paecilomyces bainier*.[20] The effect of temperature on enzyme stability is shown in Figure 4. After incubation at pH 7.0 at 20 °C for 4 h, enzyme retained full activity. When the incubation temperature was increased to 30 °C, the enzyme retained only 40% of its activity under the same conditions.

The effects of metal ions and chemical agent on Bgp3 activity was investigated (Table 2). The enzyme activity appeared to be 96% inhibited in the presence of SDS, and the enzyme did not require $Na^+$, $K^+$, $Mn^{2+}$, $Fe^{2+}$, and $Mg^{2+}$ for activity and was significantly (117%) stimulated by $Ca^{2+}$.

The substrate specificity of Bgp3 was tested using 10.0 mM *p*NP and *o*NP glycosides with *α* and *β* configurations (Table 3). The hydrolytic activity for the *p*NP and *o*NP substrates was as follows: *p*NP-*β*-D-glucopyranoside > *p*NP-*α*-L-arabinopyranoside > *o*NP-*β*-D-glucopyranoside. The enzyme exhibited no activity toward *p*NP-*α*-D-glucopyranoside, *p*NP-*α*-L-arabinofuranoside, *o*NP-*β*-D-galactopyranoside, and *p*NP-*β*-D-galactopyranoside.

**3.3. Biotransformation Pathway of Ginsenoside Rb1 by Bgp3.** The time-course experiment was performed, and the hydrolyzed products were analyzed by TLC (Figure 5). Hydrolysis of the ginsenoside Rb1 produced two different metabolites: ginsenoside Rb1 was transformed to metabolite 1, and then to metabolite 2. By the end of the reaction, almost all the ginsenoside Rb1 and metabolite 1 were transformed to metabolite 2. This indicates that metabolite 1 was an intermediate metabolite and metabolite 2 was the final product. The retention factor values of metabolites 1 and 2 on TLC were similar to those of ginsenoside Rd and compound K.

Metabolites 1 and 2 from the transformation were subjected to LC/MS to determine their $M_w$. Metabolite 1 was obtained as a white powder and displayed a pseudomolecular ion peak [M + formic acid] at $m/z$ 992 in MS-ESI, corresponding to the elemental formula $C_{48}H_{82}O_{18}$ ($M_w$, 947). Thus, metabolite 1 was confirmed as ginsenoside Rd (Figure 6a).

**Table 1. Purification Scheme for the Recombinant Protein (Bgp3)**

| purification steps | tot act. (μmol min$^{-1}$) | tot protein (mg) | sp act. (U mg$^{-1}$)[a] | purification (fold) | yield (%) |
|---|---|---|---|---|---|
| cell-free extract | 944.1 | 58.8 | 16.1 | 1 | 100 |
| first amylose affinity chromatography | 255.2 | 9.6 | 26.6 | 1.7 | 27 |
| second amylose affinity chromatography | 108.8 | 3.8 | 29.4 | 1.8 | 11.5 |

[a]One unit of Bgp3 was the amount of enzyme liberating 1 μmol min$^{-1}$ of *p*NP.


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**Table 2. Effects of Metal Ions and Chemical Agent on the Activity of Purified Recombinant Bgp3**

| metal ion or reagent (10 mM) | rel act. (%) |
|---|---|
| NaCl | 111 |
| KCl | 110 |
| $CaCO_3$ | 117 |
| $MnSO_4$ | 100 |
| $FeSO_4$, | 100 |
| $MgSO_4$ | 106 |
| SDS | 4 |
| Control | 100 |

**Table 3. Relative Activity of Purified Recombinant Bgp3 toward Various Chromogenic Substrates as Measured by *o*NP or *p*NP Release at 40 °C**

| substrate[a] | rel act.[b] (%) |
|---|---|
| *p*NP-*α*-D-glucopyranoside | 0 |
| *p*NP-*α*-L-arabinofuranoside | 0 |
| *p*NP-*β*-D-galactopyranoside | 0 |
| *p*NP-*α*-L-arabinopyranoside | 46.6 |
| *p*NP-*β*-D-glucopyranoside | 100 |
| *o*NP-*β*-D-glucopyranoside | 39.3 |
| *o*NP-*β*-D-galactopyranoside | 0 |

[a]Final concentration of each 10.0 mM. [b]The relative enzyme activity against of *p*NP-*β*-D-glucopyranoside was assumed to be 100%.

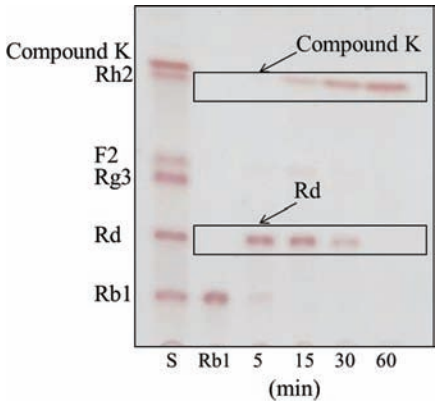


**Figure 5.** TLC analysis of the time-course transformation of ginsenoside Rb1 by Bgp3.

The molecular formula of metabolite 2 was determined to be $C_{36}H_{62}O_8$ based on the protonated molecular ion peak [M + formic acid] at $m/z$ 668 ($M_w$, 623) in MS-ESI. This result confirmed metabolite 2 was compound K (Figure 6b).

The conversion of ginsenoside Rb1 by Bgp3 was confirmed quantitatively by HPLC analysis. As shown in Figure 7, 1 mg $mL^{-1}$ ginsenoside Rb1 was transformed into 0.46 mg $mL^{-1}$ compound K after 60 min with a corresponding molar conversion yield of 77%. Hence, Bgp3 hydrolyzed the ginsenoside Rb1 along the pathway Rb1 → Rd → compound K, hydrolysis of the outer glucose molecules at position C-20, followed by hydrolysis of the inner glucose molecules at position C-3 of ginsenoside Rb1 (Figure 8). The ginsenoside-hydrolyzing *β*-D-glucosidase from *Paecilomyces bainier* sp. 229[20] exploits the hydrolytic pathway Rb1 → Rd → F2 → compound K, *β*-glucosidase from *Terrabacter ginsenosidimutans* sp.[21] exploits the hydrolytic pathway Rb1 → gypenoside XVII → gypenoside LXXV → compound K, and *β*-glycosidase from

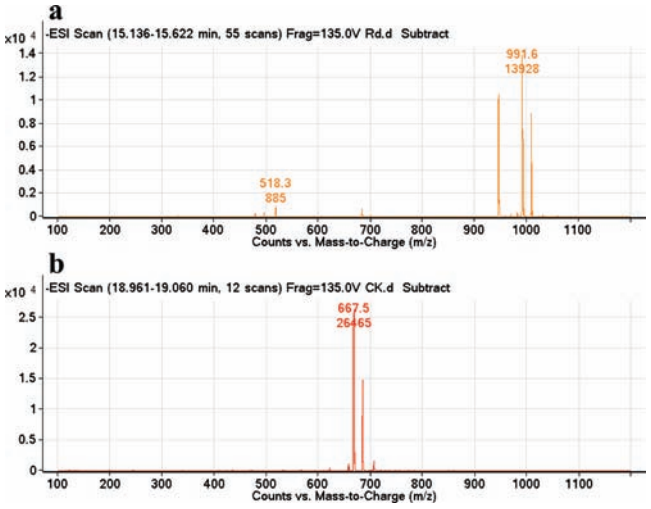


**Figure 6.** Mass spectra of ginsenoside Rb1 after hydrolysis by Bgp3. (a) Mass spectrum of Rd, $m/z$ 992 = [$M_w$ + formic acid], $M_w$ 947. (b) Mass spectrum of compound K, $m/z$ 668 = [$M_w$ + formic acid], $M_w$ 623.

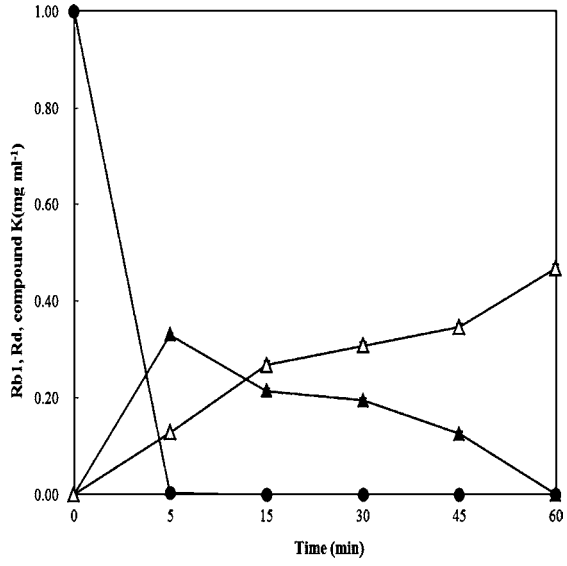


**Figure 7.** Time course for ginsenoside Rd and compound K production from ginsenoside Rb1 by Bgp3 under the optimum conditions. Rb1 (●) → Rd (▲) → compound K (Δ).

*Sulfolobus acidocaldarius*[22] exploits hydrolytic pathway Rb1 → Rd → compound K.

**3.4. Substrate Specificity of Bgp3.** Theoretically, four glucose moieties attached to the ginsenoside Rb1 could be available for hydrolysis by Bgp3, 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, Bgp3 hydrolyzed the outer glucose moiety attached to the C-20 position, and then the inner glucose moiety attached to the C-3 position of ginsenoside Rb1, effectively converted into compound K.

To investigate whether Bgp3 showed the same specificity and selectivity for glycone residues attached at the C-3 and C-20 positions of other PPD ginsenosides, the ginsenosides Rb1, Rc, Rd, and F2 were also used as substrates (Figure 9). Ginsenoside Rb1 was converted to compound K by hydrolysis of the outer glucose moiety at position C-20 and the inner glucose moiety


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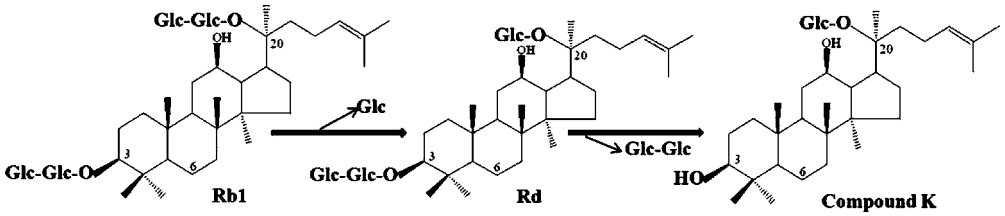


**Figure 8.** Transformation pathway from ginsenoside Rb1 to ginsenoside Rd and compound K by Bgp3.

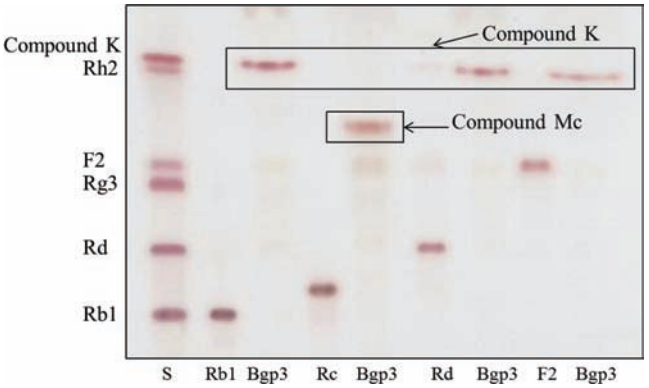


**Figure 9.** Transformation of ginsenosides Rb1, Rc, Rd, and F2 to compound Mc and compound K by Bgp3. Metabolites were analyzed by TLC.

at position C-3, while ginsenosides Rc and Rd were converted to compound Mc and compound K, respectively, by hydrolysis of the inner glucose moiety at position C-3. When ginsenoside F2 was used as a substrate, Bgp3 hydrolyzed the glucose moiety at the C-3 position of ginsenoside F2 converted to compound K. These results indicate that Bgp3 shows substrate specificity for PPD type ginsenosides that have two glucose moieties at the C-20 position, and shows specific affinity to only the outer glucose moiety attached to the C-20 position, followed by the inner glucose moiety attached to the C-3 position.

In conclusion this study demonstrated that compound K was produced from the ginsenoside Rb1 by the recombinant $\beta$-glucosidase from *Microbacterium esteraromaticum*. The recombinant $\beta$-glucosidase converted completely ginsenoside Rb1 to compound K with high productivity. Therefore the Bgp3 enzyme is considered potentially useful for the practical preparation of compound K.

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### Notes

The authors declare no competing financial interest.

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