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Cloning and Characterization of Ginsenoside-Hydrolyzing β-Glucosidase from *Lactobacillus brevis* That Transforms Ginsenosides Rb1 and F2 into Ginsenoside Rd and Compound K

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The ginsenoside-hydrolyzing β-glucosidase gene (*bgy2*) was cloned from *Lactobacillus brevis*. We expressed this gene in *Escherichia coli* BL21(DE3), isolated the resulting protein, and then utilized the enzyme for the biotransformation of ginsenosides. The *bgy2* gene contains 2,223 bp, and encodes a protein of 741 amino acids that is a member of glycosyl hydrolase family 3. β-Glucosidase (Bgy2) cleaved the outer glucose moieties of ginsenosides at the C-20 position, and the inner glucose at the C-3 position. Under optimal conditions (pH 7.0, 30°C), we used 0.1 mg/ml Bgy2 in 20 mM sodium phosphate buffer (PBS) for enzymatic studies. In these conditions, 1.0 mg/ml ginsenoside Rb1 and ginsenoside F2 were converted into 0.59 mg/ml ginsenoside Rd and 0.72 mg/ml compound K, with molar conversion productivities of 69% and 91%, respectively. In pharmaceutical and commercial industries, this recombinant Bgy2 would be suitable for producting ginsenoside Rd and compound K.

Keywords: Ginsenoside, β-glucosidase, transformation, *Lactobacillus brevis*

Introduction

Ginsenosides are the main active components in the plants of *Panax ginseng* C.A. Meyer, *Panax quinquefolius*, and *Panax notoginseng*. Ginsenosides have many reported pharmacological activities, such as anticancer [4], anti-inflammatory [18], anti-allergic [2], anti-fatigue [21], and antidiabetic activities [11]. In addition to the medicinal usage of ginseng, it is also widely consumed as a functional food in tea, powder, and capsules.

Almost 40 ginsenosides have been isolated from ginseng root. [5]. Ginsenosides that are highly present in ginseng that can be easily separated and purified include ginsenosides Rb1, Rb2, Rb3, Rc, and Rd, the major protopanaxadiol-type ginsenosides, and Re, Rf, and Rg1, the major protopanaxatriol-type ginsenosides. The minor ginsenosides, including F1, F2, Rh1, Rh2, Rg2, Rg3, and compound K, show better pharmacological effects than the major ginsenosides, although their concentrations are low or absent in ginseng [20]. Thus, various studies have focused on transforming glycosylated ginsenosides to deglycosylated ginsenosides by hydrolysis of the sugar moieties [12].

Methods, including heating [8], acid hydrolysis [3], microbial transformation, and enzymatic transformation, have been utilized to hydrolyze high-content ginsenosides into minor ginsenosides [14, 17]. Among these methods, microbial and enzymatic transformations have greater potential due to their high specificity and mild reaction conditions [9,19].

β-Glucosidase from food-grade bacteria, including *Aspergillus versicolor* and *Bacillus megaterium*, convert ginsenoside Rb1

to Rd by specifically hydrolyzing the outer glucose in the C-20 position [7,10]. Crude enzymes from *Lactobacillus paralimentarius* LH4 [13], *Leuconostoc citreum* LH1, and *L. brevis* LH8 converted ginsenoside Rb1 or Rd to compound K [14, 16]. However, the substrate specificity of these enzymes has not yet been described.

Previously, we isolated the ginsenoside-hydrolyzing *L. brevis* from kimchi, a traditional fermented food in Korea. In this study, Bgy2 from *L. brevis* exhibited selective hydrolysis for the outer C-20 position glucose and inner C-3 position glucose in ginsenosides. In pharmaceutical and commercial industries, the recombinant Bgy2 would be suitable for producing ginsenoside Rd and compound K.

Materials and Methods

Materials

L. brevis was isolated from a fermented food in Korea called kimchi [14]. Ginsenoside standards of Rb1, Rd, F2, and compound K were purchased from Chengdu Institute of Biology, CAS (China).

Cloning and Purification of Recombinant Bgy2

PCR was used to amplify the β-glucosidase gene (*bgy2*), with *L. brevis* genomic DNA as a template. The primer sequences were based on the DNA sequence of another glycosyl hydrolase (GH) 3 family domain protein from *L. brevis* (GenBank Accession No. BAN05876). We used the forward (5′-CC**C ATA TG**A CAG CCA TTC CT-3′) and reverse primers (5′-GGC C**GA ATT C**TT ATT GAA TTT GAT T-3′) to introduce NdeI and EcoRI restriction sites (boldface), respectively. We purified the PCR product and subcloned it into a pMAL-c5X plasmid digested with NdeI and EcoRI. Finally, we transformed the resulting recombinant, pMAL-bgy2, into *Escherichia coli* BL21 (DE3). Protein expression and purification were performed as previously described [17].

Characterization of Recombinant Bgy2

Under the conditions of pH 7.0 and 30°C, we used *p*NP-β-D-glucopyranoside in 20 mM PBS to determine the substrate preference of Bgy2. After 30 min, 50 mM Na_2CO_3 was added to stop the reactions. The release of *p*NP was immediately determined by measuring the absorbance of the reaction at 405 nm with a microplate reader. One unit of activity was defined as the amount of protein required to generate 1 μmol *p*NP per minute. The standard protocols for investigating the specific activity of purified Bgy2 and the effects of pH, temperature, thermostability, metals, and substrate preference on the enzyme activity were performed as previously described [17].

Enzymatic Transformation of Ginsenosides

We used ginsenoside Rb1 as a substrate to show that the existence of maltose binding protein (MBP) had no influence on enzyme activity when fused to Bgy2 in initial biotransformation experiments. Thus, under the optimal conditions (pH 7.0, 30°C), 0.1 mg/ml Bgy2 was reacted with equal volumes of ginsenosides Rb1 and F2 at 1 mg/ml in 20 mM PBS. Samples were withdrawn at regular intervals, and were extracted with equal volumes of water-saturated *n*-butanol. Subsequently, the *n*-butanol fraction was evaporated to dryness, and the methanol extract of this material was assayed via LC-MS and ESI-MS/MS.

Assay of Ginsenosides by LC-MS and ESI-MS/MS

An HPLC system (Agilent Technologies, USA) with an Agilent Poroshell ZORBAX SB-C18 column (4.6 mm × 150 mm, 5 μm) was used to separate samples under a constant flow rate of 0.5 ml/min (25°C). Water (A) and acetonitrile (B) were used for the mobile phase, with a gradient elution of 23–68% (B) from 0 to 15 min, 68–68% (B) from 15 to 22 min, and 68–100% (B) from 22 to 32 min. The injection volume was 5 μl for each sample.

For determining and confirming the presence of ginsenosides, we used negative ESI-MS/MS. All mass spectrometric experiments were performed on a triple-quadrupole tandem mass spectrometer (Agilent Technologies, USA). The capillary voltage was set to 3,000 V for the negative mode; the gas flow rate was 3 l/min, and the gas temperature was 300°C.

Results and Discussion

Expression and Purification of Recombinant Bgy2

The *bgy2* gene contains 2,223 bp and encodes a protein of 741 amino acids that is a member of GH family 3. We amplified the gene by PCR and then inserted it into the pMAL-c5X vector. We tested various conditions for protein induction to maximize the production of the fusion protein. Induction under 16°C for 12 h with 0.2 mM IPTG showed maximal production of a soluble active fusion enzyme. MBP-Bgy2 was purified by MBP-bound amylose resin, and then the supernatant from the cell lysates and the purified enzyme were analyzed by SDS-PAGE. The molecular mass of the MBP-Bgy2 predicted from the amino acid sequence was 123 kDa, which was the mass observed by SDS-PAGE (Fig. 1).

The other proteins belonging to GH family 3 include β-glycosidase from *Terrabacter ginsenosidimutans* sp. [1], which converted ginsenoside Rb1 via hydrolysis of the two C-3 glucose moieties to produce gypenoside LXXV, and then the C-20 outer glucose moieties were hydrolyzed to produce compound K. β-Glycosidase from *Flavobacterium johnsoniae* and *Microbacterium esteraromaticum* converted ginsenoside Rb1 to Rg3 via hydrolysis of the two glucoses at the C-20 position [6, 14].

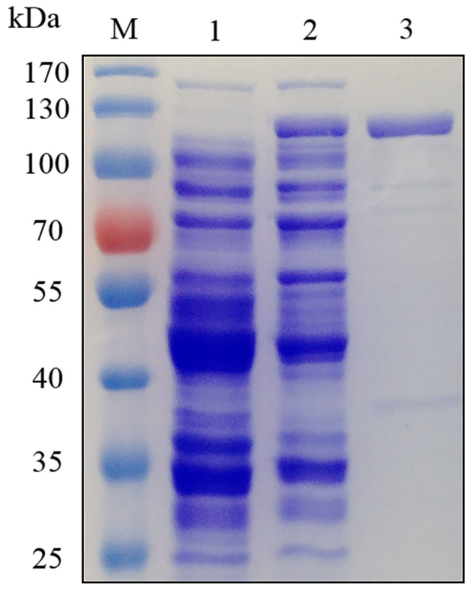

Fig. 1. Purification of recombinant Bgy2.
M, molecular mass markers; lane 1, crude extract of uninduced BL21 (DE3) cells carrying pMAL-Bgy2; lane 2, crude extract of induced recombinant BL21 (DE3) cells carrying pMAL-Bgy2; lane 3, amylose affinity-purified MBP-Bgy2.

Table 1. Effects of metal ions on the activity of recombinant Bgy2.

Metal ion (10 mM)	Relative activity ± SD (%)
NaCl	89 ± 2
KCl	90 ± 1
$CaCO_3$	99 ± 6
$MnSO_4$	81 ± 11
$MgSO_4$	87 ± 6
$Fe_2(SO_4)_3$	77 ± 4
Control	100 ± 1

Enzyme Characterization

Recombinant Bgy2 had optimal activity in 20 mM PBS at pH 7.0 (Fig. 2A), and the optimal temperature was 30°C (Fig. 2B). We investigated the influence of temperature on enzyme activity by evaluating the activity from 20°C to 70°C (pH 7.0). Enzyme activity was relatively stable from 20°C to 40°C, but above 50°C, the activity was completely lost (Fig. 2B).

We also examined the influence of metal ions on Bgy2 activity (Table 1). Bgy2 did not require Na^+, K^+, Ca^{2+}, Mn^{2+}, Mg^{2+}, or Fe^{3+} for activity.

We used 10.0 mM *p*NP and *o*NP glycosides with α and β

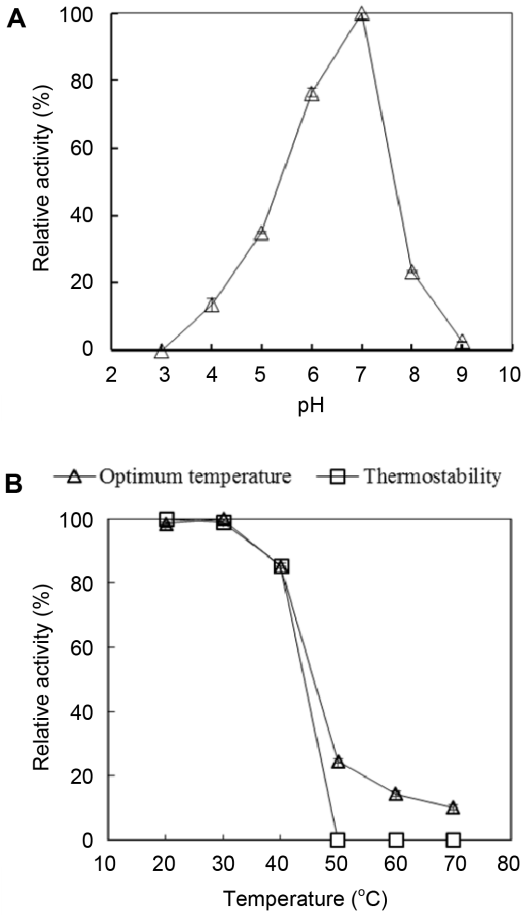

Fig. 2. Characterization of recombinant Bgy2.
(**A**) Effect of pH on the activity of recombinant Bgy2 determined using *p*NP-β-D-glucopyranoside as a substrate. The following buffers (20 mM) were tested: citric acid/Na_2HPO_4 buffer (pH 3.0–5.0), sodium phosphate buffer (pH 6.0–8.0), and glycine/NaOH buffer (pH 9.0–10.0). The maximum activity observed at pH 7.0 was taken as 100% = 96 U (mg protein)$^{-1}$. (**B**) Effect of temperature on the stability and activity of recombinant Bgy2 determined using *p*NP-β-D-glucopyranoside as a substrate. Open triangle, the thermo-dependence of enzyme activity was assayed in 20 mM sodium phosphate buffer (pH 7.0) at various temperatures ranging from 20°C to 70°C. The maximum activity observed at 30°C was taken as 100% = 96 U (mg protein)$^{-1}$. Open square, thermostability was tested by incubating aliquots of the enzyme in 20 mM sodium phosphate buffer (pH 7.0) for 30 min at different temperatures. Data represent the means of three experiments, and error bars represent the standard deviation.

configurations to examine the substrate specificity of Bgy2. The results are shown in Table 2, illustrating that Bgy2 has high activity toward *p*NP-β-D-glucopyranoside, and less activity toward *o*NP-β-D-glucopyranoside. Bgy2 showed no activity for *p*NP-α-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-L-arabinofuranoside, or *o*NP-

Table 2. Relative activity of recombinant Bgy2 toward various chromogenic substrates as measured by *o*NP or *p*NP release at 30°C.

Substrate[a]	Relative activity ± SD (%)[b]
*p*NP-α-D-glucopyranoside	0
*p*NP-α-L-arabinofuranoside	0
*p*NP-β-D-galactopyranoside	0
*p*NP-β-D-glucopyranoside	100 ± 3
*o*NP-β-D-glucopyranoside	93 ± 2
*o*NP-β-D-galactopyranoside	0

[a]Final concentration of each was 10.0 mM.

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

β-D-galactopyranoside.

Biotransformation Pathway of Ginsenosides Rb1 and F2 by Bgy2

We performed a time-course analysis of the enzymatic reaction, and then analyzed the metabolites via LC-MS and ESI-MS/MS. Hydrolysis of the ginsenosides Rb1 and F2 yielded two distinct metabolites: ginsenoside Rb1 was converted to metabolite 1, and ginsenoside F2 was converted to metabolite 2 (Figs. 3A and 3B).

ESI-MS/MS was used to obtain structural information about metabolites 1 and 2. In the ESI-MS/MS spectrum of metabolite 1, an ion at *m/z* 945.5 was yielded via loss of the HCOOH (46 Da) from the $[M+HCOO]^-$ ion at *m/z* 991.6, and fragmentations at *m/z* 783.4 and 621.4 were also

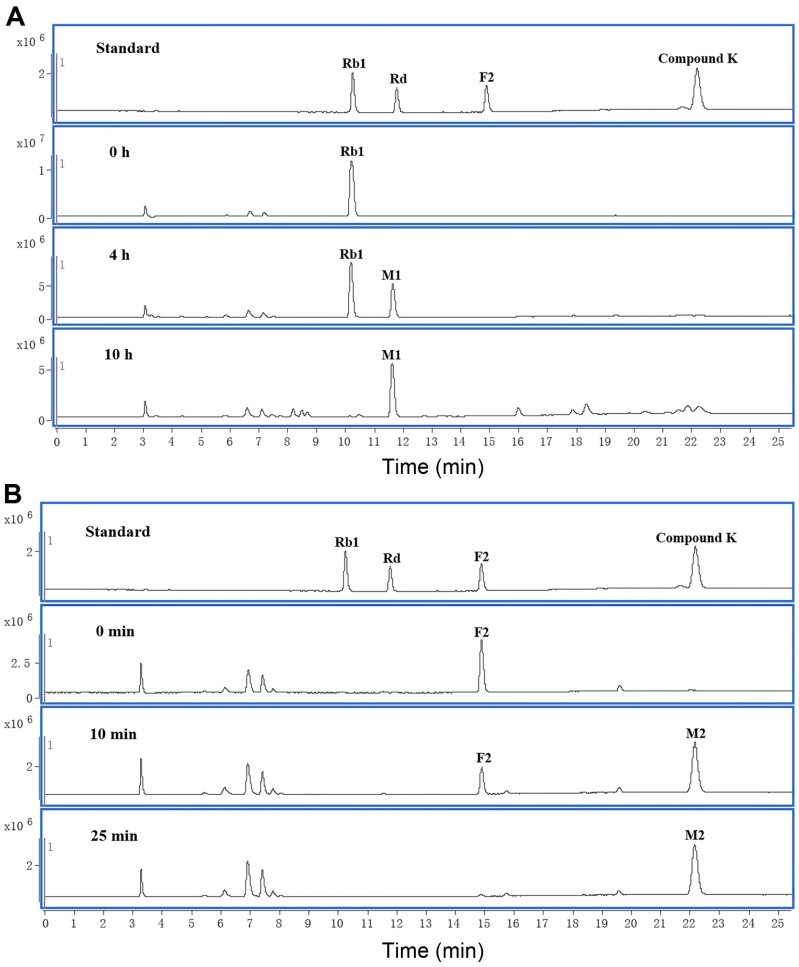

Fig. 3. Mass spectrometry analyses of the transformation of ginsenosides Rb1 and F2 by Bgy2.
(**A**) LC-MS analysis of the time course of the transformation of ginsenoside Rb1 by Bgy2 from *L. brevis*. (**B**) LC-MS analysis of the time course of the transformation of ginsenoside F2 by Bgy2 from *L. brevis*. (**C**) The MS/MS fragmentation spectrum of ginsenoside Rd using ESI-MS/MS. (**D**) The MS/MS fragmentation spectrum of M1 using ESI-MS/MS. (**E**) The MS/MS fragmentation spectrum of compound K using ESI-MS/MS. (**F**) The MS/MS fragmentation spectrum of M2 using ESI-MS/MS. M1, metabolite 1; M2, metabolite 2.

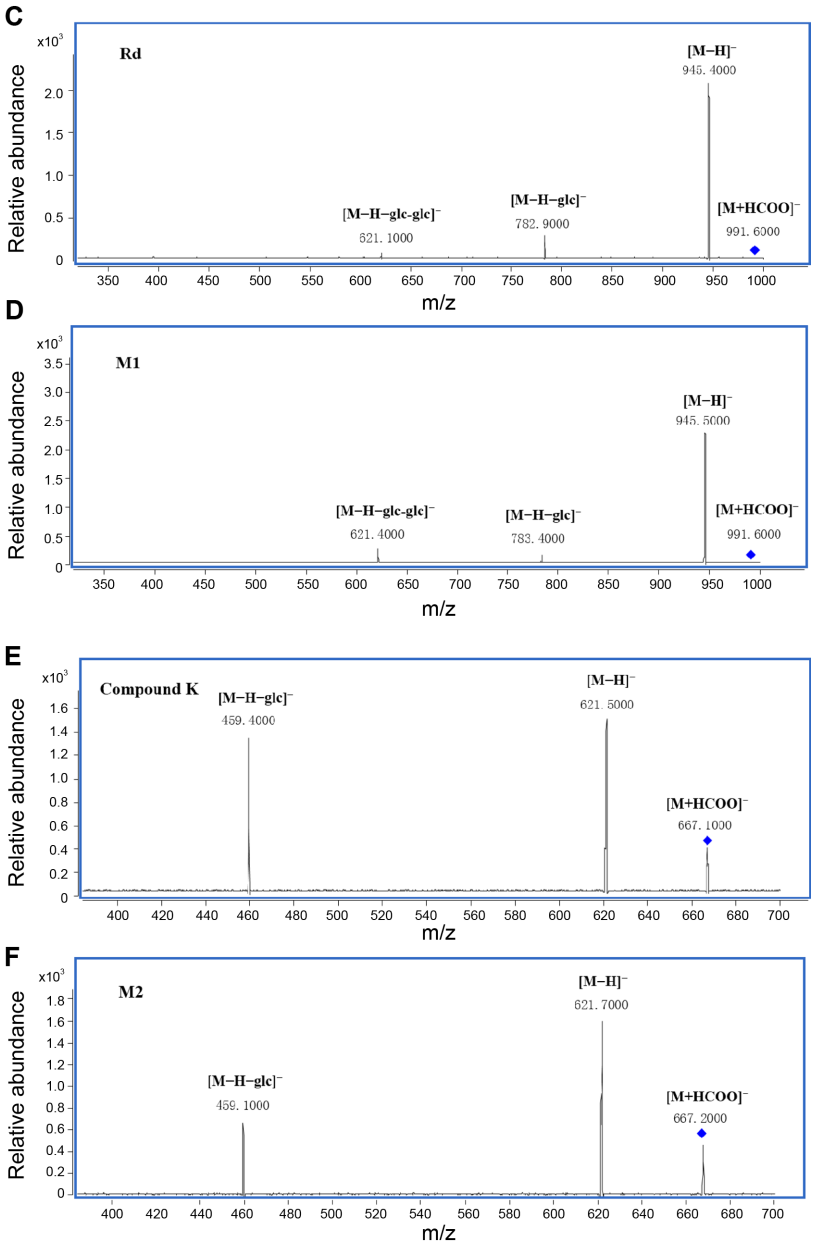

Fig. 3. Continued.

generated (Fig. 3D). When compared with the reference standard ginsenoside Rd, metabolite 1 had an equal retention time and ESI-MS/MS fragmentation patterns (Figs. 3A and 3C). Therefore, metabolite 1 was identified as ginsenoside Rd.

The ESI-MS/MS spectrum of metabolite 2 is displayed in Fig. 3F. The ion at *m/z* 621.7 was from the loss of HCOOH (46 Da) from the $[M+HCOO]^-$ ion at *m/z* 667.2. An $[aglycone–H]^-$ ion at *m/z* 459.1 was detected by loss of a glucose residue (162 Da), demonstrating that the aglycone is of the PPD type. When compared with the reference standard compound K, metabolite 2 had an equal retention time and ESI-MS/MS fragmentation patterns (Figs. 3B and 3E). Therefore, metabolite 2 was identified as compound K.

The conversion of ginsenosides Rb1 and F2 via Bgy2 was identified quantitatively via LC-MS/MS analysis. A total of 1.0 mg/ml ginsenoside Rb1 was transformed into 0.59 mg/ml ginsenoside Rd in 10 h, with a corresponding molar conversion productivity of 69%, and 1.0 mg/ml ginsenoside F2 was transformed into 0.72 mg/ml compound K within

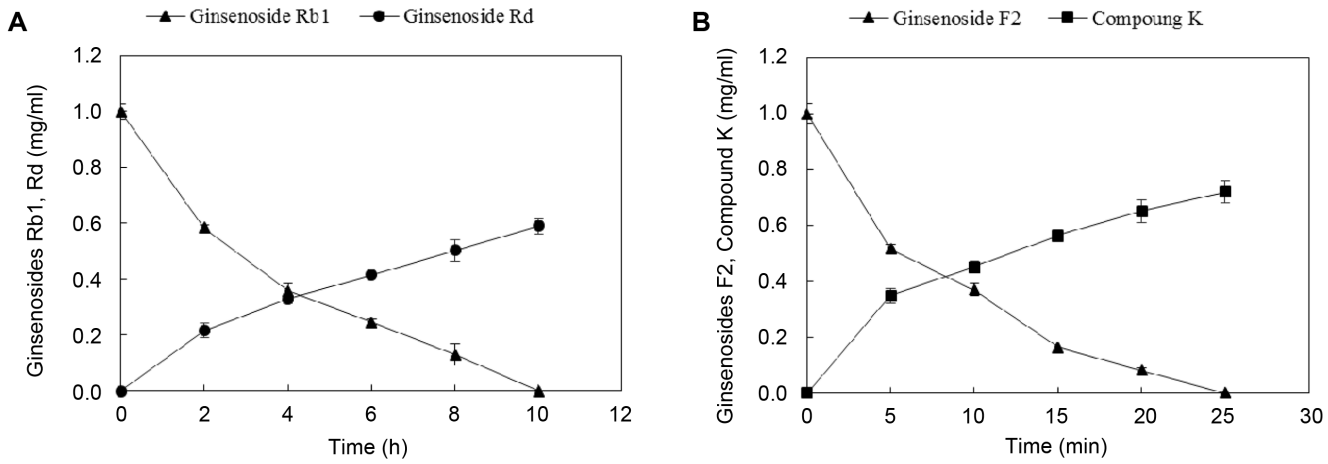

Fig. 4. Conversion of ginsenosides Rb1 and F2 to Rd and compound K, respectively, by Bgy2 from *L. brevis*.
(**A**) Conversion of ginsenoside Rb1 (filled triangle) to Rd (filled circles). (**B**) Conversion of ginsenoside F2 (filled triangle) to compound K (filled squares).

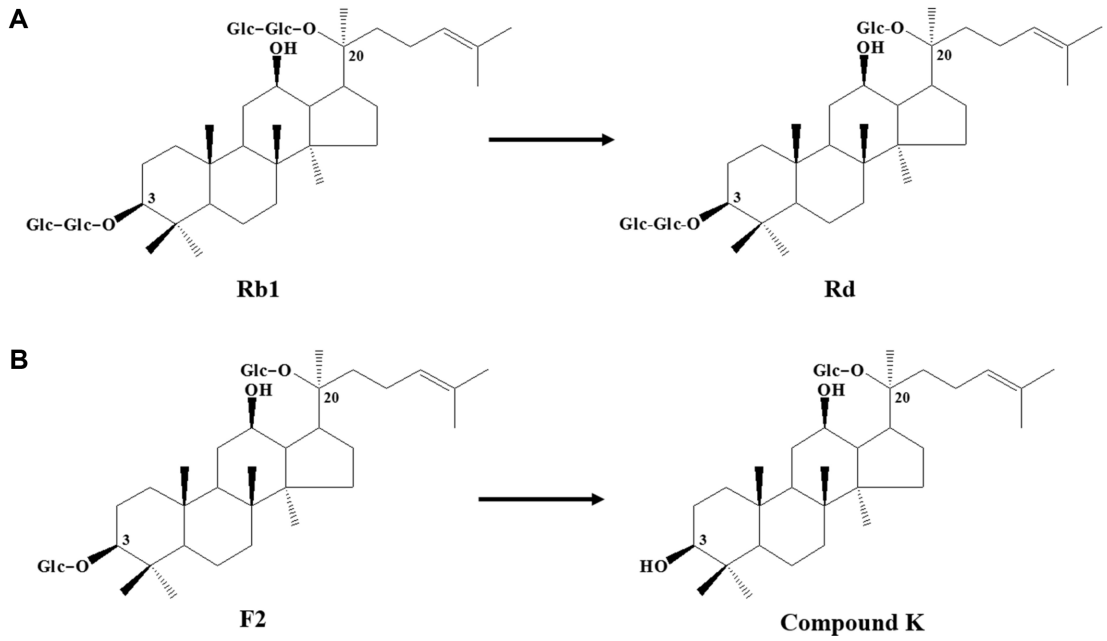

Fig. 5. Transformation pathways of ginsenosides Rb1 and F2 by Bgy2 from *L. brevis*.

25 min, with a corresponding molar conversion productivity of 91% (Fig. 4).

Therefore, Bgy2 exhibits substrate specificity for ginsenosides Rb1 and F2 with glucose moieties at the C-20 and C-3 positions, and illustrates specific affinity to the outer C-20 glucose and C-3 glucose. The transformation pathway illustrated in Fig. 5 depicts the hydrolysis of ginsenosides Rb1 and F2 to ginsenoside Rd and compound K, respectively, via Bgy2 (Fig. 5). The hydrolytic pathway of the crude enzyme from *L. brevis* LH8 is Rd → F2 → compound K by cleaving the outer and inner glucose moieties at position C-3 of ginsenoside Rd [14].

In conclusion, Bgy2 is a member of the GH 3 family from *L. brevis*. Bgy2 hydrolyzed the outer C-20 glucose and inner C-3 glucose in PPD-type ginsenosides, but not the inner C-20 glucose position and the outer glycoside linked to glucose at the C-3 position. Bgy2 can transform ginsenosides Rb1 and F2 into ginsenoside Rd and compound K,

respectively. Therefore, in pharmaceutical and commercial industries, this recombinant Bgy2 would be suitable for producing ginsenoside Rd and compound K.

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References

1. An DS, Cui CH, Lee HG, Wang L, Kim SC, Lee ST, *et al*. 2010. Identification and characterization of a novel *Terrabacter ginsenosidimutans* sp. nov. β-glucosidase that transforms ginsenoside Rb1 into the rare gypenosides XVII and LXXV. *Appl. Environ. Microbiol*. **76:** 5827-5836.
2. Bae EA, Choo MK, Park EK, Park SY, Shin HY, Kim DH. 2002. Metabolism of ginsenoside Rc by human intestinal bacteria and its related antiallergic activity. *Biol. Pharm. Bull*. **25:** 743-747.
3. Bae EA, Han MJ, Kim EJ, Kim DH. 2004. Transformation of ginseng saponins to ginsenoside Rh2 by acids and human intestinal bacteria and biological activities of their transformants. *Arch. Pharm. Res*. **27:** 61-67.
4. Chae S, Kang KA, Chang WY, Kim MJ, Lee SJ, Lee YS, *et al*. 2009. Effect of compound K, a metabolite of ginseng saponin, combined with gamma-ray radiation in human lung cancer cells in vitro and in vivo. *J. Agric. Food Chem*. **57:** 5777-5782.
5. Cheng LQ, Na JR, Kim MK, Bang MH, Yang DC. 2007. Microbial conversion of ginsenoside Rb1 to minor ginsenoside F2 and gypenoside XVII by *Intrasporangium* sp. GS603 isolated from soil. *J. Microbiol. Biotechnol*. **17:** 1937-1943.
6. Kim JK, Cui CH, Liu Q, Yoon MH, Kim SC, Im WT. 2013. Mass production of the ginsenoside Rg3(S) through the combinative use of two glycoside hydrolases. *Food Chem*. **141:** 1369-1377.
7. Kim MK, Lee JW, Lee KY, Yang DC. 2005. Microbial conversion of major ginsenoside Rb(1) to pharmaceutically active minor ginsenoside Rd. *J. Microbiol*. **43:** 456-462.
8. Kim WY, Kim JM, Han SB, Lee SK, Kim ND, Park MK, *et al*. 2000. Steaming of ginseng at high temperature enhances biological activity. *J. Nat. Prod*. **63:** 1702-1704.
9. Lee SJ, Kim Y, Kim MG. 2015. Changes in the ginsenoside content during the fermentation process using microbial strains. *J. Ginseng Res*. **39:** 392-397.
10. Lin F, Guo X, Lu W. 2015. Efficient biotransformation of ginsenoside Rb1 to Rd by isolated *Aspergillus versicolor*, excreting β-glucosidase in the spore production phase of solid culture. *Antonie Van Leeuwenhoek* **108:** 1117-1127.
11. Ni HX, Yu NJ, Yang XH. 2010. The study of ginsenoside on PPARgamma expression of mononuclear macrophage in type 2 diabetes. *Mol. Biol. Rep*. **37:** 2975-2979.
12. Park CS, Yoo MH, Noh KH, Oh DK. 2010. Biotransformation of ginsenosides by hydrolyzing the sugar moieties of ginsenosides using microbial glycosidases. *Appl. Microbiol. Biotechnol*. **87:** 9-19.
13. Quan LH, Kim YJ, Li GH, Choi KT, Yang DC. 2013. Microbial transformation of ginsenoside Rb1 to compound K by *Lactobacillus paralimentarius*. *World J. Microbiol. Biotechnol*. **29:** 1001-1007.
14. Quan LH, Liang Z, Kim HB, Kim SH, Kim SY, Noh YD, Yang DC. 2008. Conversion of ginsenoside Rd to compound K by crude enzymes extracted from *Lactobacillus brevis* LH8. *J. Ginseng Res*. **32:** 226-231.
15. Quan LH, Min JW, Yang DU, Kim YJ, Yang DC. 2012. Enzymatic biotransformation of ginsenoside Rb1 to 20(S)-Rg3 by recombinant β-glucosidase from *Microbacterium esteraromaticum*. *Appl. Microbiol. Biotechnol*. **94:** 377-384.
16. Quan LH, Piao JY, Min JW, Yang DU, Lee HN, Yang DC. 2011. Bioconversion of ginsenoside Rb1 into compound K by *Leuconostoc citreum* LH1 isolated from kimchi. *Braz. J. Microbiol*. **42:** 1227-1237.
17. Quan LH, Wang C, Jin Y, Wang TR, Kim YJ, Yang DC. 2013. Isolation and characterization of novel ginsenoside-hydrolyzing glycosidase from *Microbacterium esteraromaticum* that transforms ginsenoside Rb2 to rare ginsenoside 20(*S*)-Rg3. *Antonie Van Leeuwenhoek* **104:** 129-137.
18. Wu JY, Gardner BH, Murphy CI, Seals JR, Kensil CR, Recchia J. 1992. Saponin adjuvant enhancement of antigen-specific immune responses to an experimental HIV-1 vaccine. *J. Immunol*. **148:** 1519-1525.
19. Xie J, Zhao D, Zhao L, Pei J, Xiao W, Ding G, *et al*. 2016. Characterization of a novel arabinose-tolerant α-L-arabinofuranosidase with high ginsenoside Rc to ginsenoside Rd bioconversion productivity. *J. Appl. Microbiol*. **120:** 647-660.
20. Xu QF, Fang XL, Chen DF. 2003. Pharmacokinetics and bioavailability of ginsenoside Rb1 and Rg1 from *Panax notoginseng* in rats. *J. Ethnopharmacol*. **84:** 187-192.
21. 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.