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ORIGINAL RESEARCH PAPER

Biotransformation of gypenoside XVII to compound K by a recombinant β-glucosidase

Fei-Liang Zhong · Wei-Wei Dong · Songquan Wu · Jun Jiang · Deok-Chun Yang · Donghao Li · Lin-Hu Quan

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Abstract
Objective To study the *β*-glucosidase gene (*bgy1*) from *Lactobacillus brevis* that was cloned and expressed in *Escherichia coli* BL21 (DE3) and then using it for the biotransformation of gypenoside XVII.
Results The *bgy1* gene consists of 2283 bp encoding 761 amino acids, with homology to the glycosyl hydrolase family-3 protein domain. The enzyme (Bgy1) hydrolyzed the glucose moieties at the C-3 position and the outer glucose moieties at the C-20 position of gypenoside XVII. Using 0.1 mg enzyme ml^{-1} in 20 mM sodium phosphate buffer at 30 °C and pH 6.0, 1 mg gypenoside XVII ml^{-1} was transformed into 0.58 mg compound K ml^{-1} within 6 h, with a corresponding molar conversion yield of 89 %.
Conclusion The recombinant Bgy1 is considered potentially useful for the practical preparation of compound K.

Keywords Biotransformation · Compound K · β-Glucosidase · Gypenoside XVII

Introduction

Panax quinquefolius (American ginseng) is used as a traditional medicine for its various functions, including anti-tumor (Wakabayashi et al. 1997), anti-inflammatory (Bae et al. 2002) and anti-aging effects (Cho et al. 2006). The ginseng root consists of ginsenosides, polysaccharides, peptides, polyacetyl enic alcohols, and fatty acids. The ginsenosides are the main components responsible for these beneficial functions. More than 40 ginsenosides have been isolated from ginseng root (Cheng et al. 2007). Pharmaceutically-active ginsenosides exist as deglycosylated forms at low concentrations or are absent in ginseng (Tawab et al. 2003). These deglycosylated ginsenosides, including ginsenosides Rg3, Rh2, and compound K, can be produced by the hydrolysis of sugar moieties from the major ginsenosides (Rb1, Rb2, Rc, Rd and gypenoside XVII) (Noh et al. 2009).

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F.-L. Zhong · W.-W. Dong · S. Wu · J. Jiang · D. Li (✉) · L.-H. Quan (✉)
Key Laboratory of Natural Resources of Changbai Mountain and Functional Molecules, Ministry of Education, Yanbian University, Park Road 977, Yanji 133002, Jilin, People's Republic of China
e-mail: dhli@ybu.edu.cn

L.-H. Quan
e-mail: lhquan@ybu.edu.cn

D.-C. Yang (✉)
Department of Oriental Medicinal Material and Processing, College of Life Science, Korean Ginseng Center Most Valuable Product and Ginseng Genetic Resource Bank, Kyung-Hee University, Seocheon-dong, Giheung-gu, Yongin-Si, Gyeonggi-do 446-701, Republic of Korea
e-mail: dcyang@khu.ac.kr

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Deglycosylated ginsenosides can be produced by hydrolyzing the sugar moieties linked to the C-3, C-6, and C-20 positions in major glycosylated ginsenosides by heating (Kim et al. 2000), acid treatment (Bae et al. 2004), fermentation (Quan et al. 2008), or enzymatic conversion methods (Quan et al. 2012b). Among these, enzymatic conversion is the most promising method for the preparation of diverse deglycosylated ginsenosides via the selective hydrolysis of the sugar moieties of ginsenosides because of its high specificity, yield, and productivity.

Major ginsenosides Rb1, Rb2, Rc and gypenoside XVII are metabolized to compound K (see Fig. 1) by human intestinal microbes which is absorbed into the blood (Lee et al. 2009; Wan et al. 2013). Compound K exhibits anti-tumor, anti-inflammatory, and anti-allergic actions more potent than those of the major ginsenosides. Major ginsenosides in *P. quinquefolius* (American ginseng) include ginsenosides Rb1, Rb2, Rb3, Rd, Re, Rg1 and gypenoside 1XVII (Wan et al. 2013). Because gypenoside XVII has three glucose moieties, hydrolyzing one glucose moiety at C-3 and C-20 can effectively transformed the gypenoside XVII into the more pharmacologically-active minor ginsenoside, compound K (Fig. 1).

In this study, the glycosyl hydrolase family 3 ginsenoside-hydrolyzing glycosidase gene (*bgy1*) from *Lactobacillus brevis* was cloned and expressed in *Escherichia coli* BL21 (DE3). The enzyme (Bgy1) hydrolyzed the glucose moieties at the C-3 position and the outer glucose moieties at the C-20 position of gypenoside XVII, effectively converting it into gypenoside LXXV and compound K.

Materials and methods

Materials

Reference standards of ginsenosides Rb1, Rd, F2, compound K, and gypenoside XVII were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). *Lactobacillus brevis* was isolated from kimchi a traditional Korean fermented food (Quan et al. 2008).

Analysis of ginsenosides by LC–MS

Chromatographic analysis was performed using an Agilent 1260 series LC system (Agilent Technologies, USA) with an Agilent Poroshell Zorbax SB-C_{18} column (4.6 mm × 150 mm, 5 μm) at 25 °C. The mobile phase, at 0.5 ml min^{-1}, was water (A) and acetonitrile (B), using a gradient elution of 23–68 % (B) at 0–15 min, 68–68 % (B) at 15–22 min, and 68–100 % (B) at 22–32 min. The sample volume injected was set. Five μl of sample was injected. Data was processed using Mass Hunter Acquisition Software, Version B.07.00 (Agilent Technologies, USA).

Positive ESI–MS was applied to detect and confirm the presence of the ginsenosides. All mass spectrometric experiments were performed on a triple-quadrupole tandem mass spectrometer (Agilent Technologies, USA). The capillary voltage was set to 4000 V for positive mode and to 3000 V for negative mode; the gas flow rate was 3 l min^{-1}, and at 300 °C. Full-scan mass spectra were acquired in a mass range of *m/z* 100–1500. The masses of the positive-ion fragments $[M + Na]^+$ of potential metabolites were examined to analyze the ginsenosides.

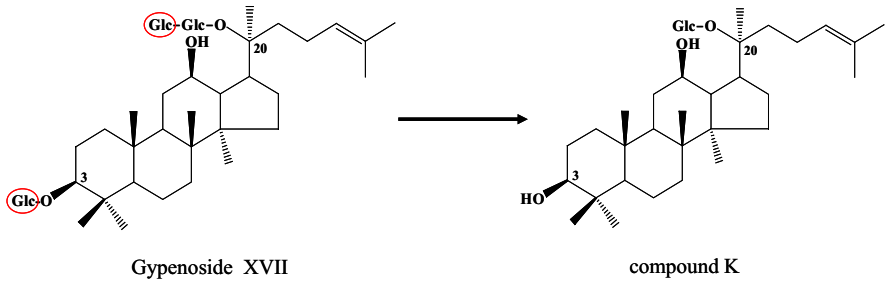

Fig. 1 Target reaction scheme: deglycosylation of gypenoside XVII to compound K

Molecular cloning, expression, and purification of recombinant Bgy1

Genomic DNA from *Lactobacillus brevis* was extracted using a genomic DNA extraction kit (Gene ALL, Korea). The gene, *bgy1* (GenBank accession number BAN07577) encoding the ginsenoside hydrolase, was amplified by PCR using the following primers (with *Nde*I and *Eco*RI restriction sites in boldface): *bgy1*F (5′-CC**C ATA TG**A ACA AAA AGA AAG GA-3′) and *bgy1*R (5′-GGC C**GA ATT C**TT ATT GAC GTA A-3′). The amplified DNA fragment was purified, digested with *Nde*I and *Eco*RI and inserted into pMAL-c5X vector to generate a maltose-binding protein (MBP)-*bgy1* gene fusion. The Bgy1 protein expression construct was verified by DNA sequencing. The recombinant pMAL-bgy1 was transformed into *Escherichia coli* BL21(DE3), which was grown in LB/ampicillin medium at 37 °C with shaking at 190 rpm until the culture reached an OD_{600} of 0.6.

Protein expression was induced by adding 0.2 mM IPTG. The transformed *E. coli* was incubated for an additional 10 h at 175 rpm at 20 °C then harvested by centrifugation at 5000×*g* for 30 min at 4 °C. The cells were washed twice with 20 mM sodium phosphate buffer (pH 6.0, 1 mM EDTA, and 1 mM NaCl) and then resuspended in 20 mM sodium phosphate buffer (pH 6.0). Cells were disrupted ultrasonically at 0 °C. Unbroken cells and cell debris were removed by centrifugation (12,000×*g*, at 4 °C for 30 min). The crude extract (5 ml) was applied to a pre-equilibrated amylase column (5 ml, NEB, USA), followed by washing with 12 column volumes of elution buffer (20 mM sodium phosphate buffer pH 6.0, 1 mM EDTA, and 1 mM NaCl). The bound proteins were eluted with elution buffer supplemented with 10 mM maltose. Protein concentration was determined using the SMART BCA protein assay kit (Intron, Korea).

Characterization of recombinant Bgy1

The specific activity of the purified Bgy1 was determined using *p*NP-*β*-D-glucopyranoside as a substrate in 20 mM sodium phosphate buffer, pH 6.0, at 30 °C. Reactions were stopped after 30 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 *p*NP per min. The standard reaction to investigate the specific activity of purified Bgy1 and the effects of pH, temperature and thermostability on the enzyme activity was performed as previously described (Quan et al. 2012b).

Enzymatic hydrolysis of ginsenosides

Initial biotransformation experiments using gypenoside XVII as a substrate showed that the presence of MBP fused to Bgy1 did not affect the enzyme activity. Therefore, the fused enzyme at 0.1 mg ml^{-1} dissolved in 20 mM sodium phosphate buffer (pH 6.0) was incubated with an equal volume of gypenoside XVII at 1 mg ml^{-1} in 20 mM sodium phosphate buffer (pH 6.0) at 30 °C. Samples were withdrawn at regular intervals, and were extracted with an equal volume of water-saturated *n*-butanol; subsequently, the *n*-butanol fraction was evaporated to dryness, and the methanol extract was assayed by LC–MS.

Results and discussion

Expression and purification of recombinant Bgy1

A gene consists of 2283 bp encoding 761 amino acids, with homology to the protein domain of the glycosyl hydrolase family 3. This gene, *bgy1*, was amplified via PCR and then inserted into the pMAL-c5X vector and expressed in *E. coli* BL21 (DE3). Bgy1 is homologous to the glycosyl hydrolase family 3 based on its amino acid sequence similarity to the β-glycosidases in *Microbacterium esteraromaticum* (43 %, GenBank accession number JN 603820), *Pseudonocardia* sp. Gsoil 1536 (41 %, GenBank accession number JX960416) and *Microbacterium esteraromaticum* (40 %, GenBank accession number JN 603821). The characterized ginsenoside-hydrolyzing glycoside hydrolase in family 3 enable the transformation of major ginsenosides: Rb1 → Rd → compound K (Quan et al. 2012a), Rb1 → Rd → Rg3 (Quan et al. 2012c), Rg1 → Rh1 and Re → Rg2 (Quan et al. 2012b).

To maximize the yield of the fusion protein, we tested the protein induction under different conditions. Induction with 0.2 mM IPTG at 20 °C for 10 h produced the maximum level of soluble active fusion enzyme. The MBP-Bgy1 fusion protein was purified

by MBP-bind amylose resin, and then the supernatant from the cell lysates as well as the purified protein was applied to SDS-PAGE. The molecular mass of the MBP-Bgy1 calculated via an amino acid sequence was 126 kDa, which is similar to the mass detected in SDS-PAGE (Fig. 2a).

Enzyme characterization

The recombinant Bgy1 had optimal activity at pH 6.0 (Fig. 2b) and at 30 °C (Fig. 2c). Activity was completely lost at 60 °C (Fig. 2c).

Biotransformation pathway of gypenoside XVII by Bgy1

A time-course experiment was performed, and the hydrolyzed products were analyzed by LC–MS. Gypenoside XVII and its metabolites were identified by comparing the retention times and accurate masses with those of standard compounds. Detection of the $[M + Na]^+$ ions was used to identify the metabolites. The hydrolysis of gypenoside XVII produced two different sequential metabolites: metabolite 1, then metabolite 2. By the end of the reaction, almost all of the gypenoside XVII and metabolite 1 were transformed to metabolite 2 within 6 h. This indicates that metabolite 1 is an intermediate metabolite and metabolite 2 is the final product (Fig. 3a). Figure 3b shows the extracted ion chromatograms (EIC) of gypenoside XVII for the $[M + Na]^+$ ion at *m/z* 969 and its deglycosylated metabolite 1 (15.9 min) at *m/z* 807 and metabolite 2 (21.1 min) at *m/z* 645. From the retention times and accurate mass comparisons with standard compounds, metabolite 2 was identified as compound K.

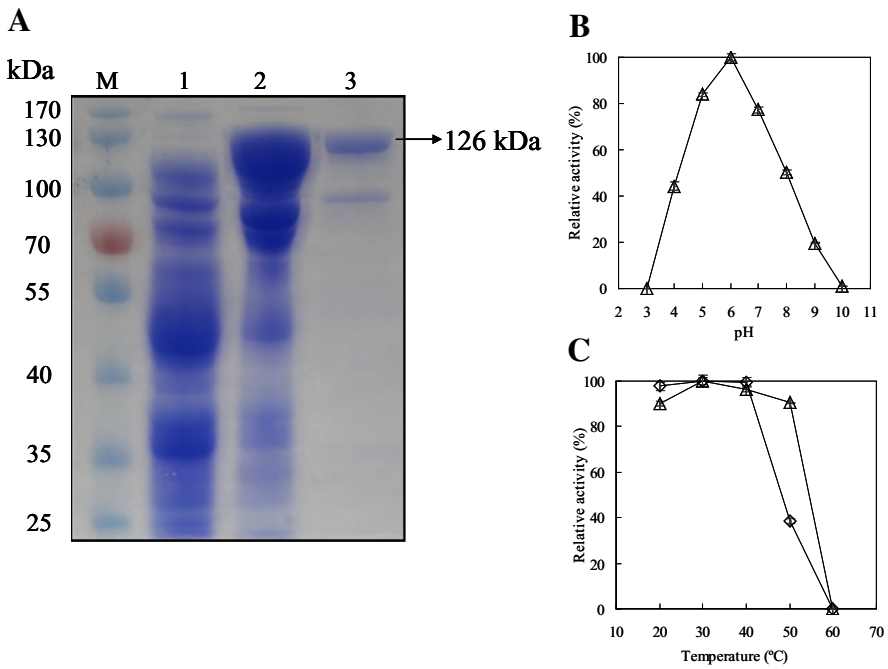

Fig. 2 **a** Purification of recombinant Bgy1. *M* molecular mass markers; *lane 1* crude extract of uninduced BL21 (DE3) cells carrying pMAL-Bgy1; *lane 2* crude extract of induced recombinant BL21 (DE3) cells; *lane 3* amylose affinity purified pMAL-Bgy1. **b** Effect of pH on the activity of recombinant Bgy1 determined using *p*NP-*β*-D-glucopyranoside as a substrate. The following buffers (20 mM) were used: citric acid/Na_2HPO_4 buffer (pH 3–5), sodium phosphate buffer (pH 6–8), and glycine/NaOH buffer (pH 9–10). Maximum activity at pH 6.0 was taken as 100 % = 89 U mg protein^{-1}. **C** Effect of temperature on the stability and activity of recombinant Bgy1 determined using *p*NP-*β*-D-glucopyranoside as a substrate. Δ, the thermo-dependence of enzyme activity was assayed in 20 mM sodium phosphate buffer (pH 6.0) at various temperatures ranging from 20 to 60 °C (*open triangle*). Maximum activity observed at 30 °C was taken as 100 % = 89 U mg protein^{-1}. Thermostability was tested by incubating aliquots of the enzyme in 20 mM sodium phosphate buffer (pH 6.0) for 30 min at different temperatures (*open quadrangle*). Data represent the means of three experiments, and error bars represent the standard deviation

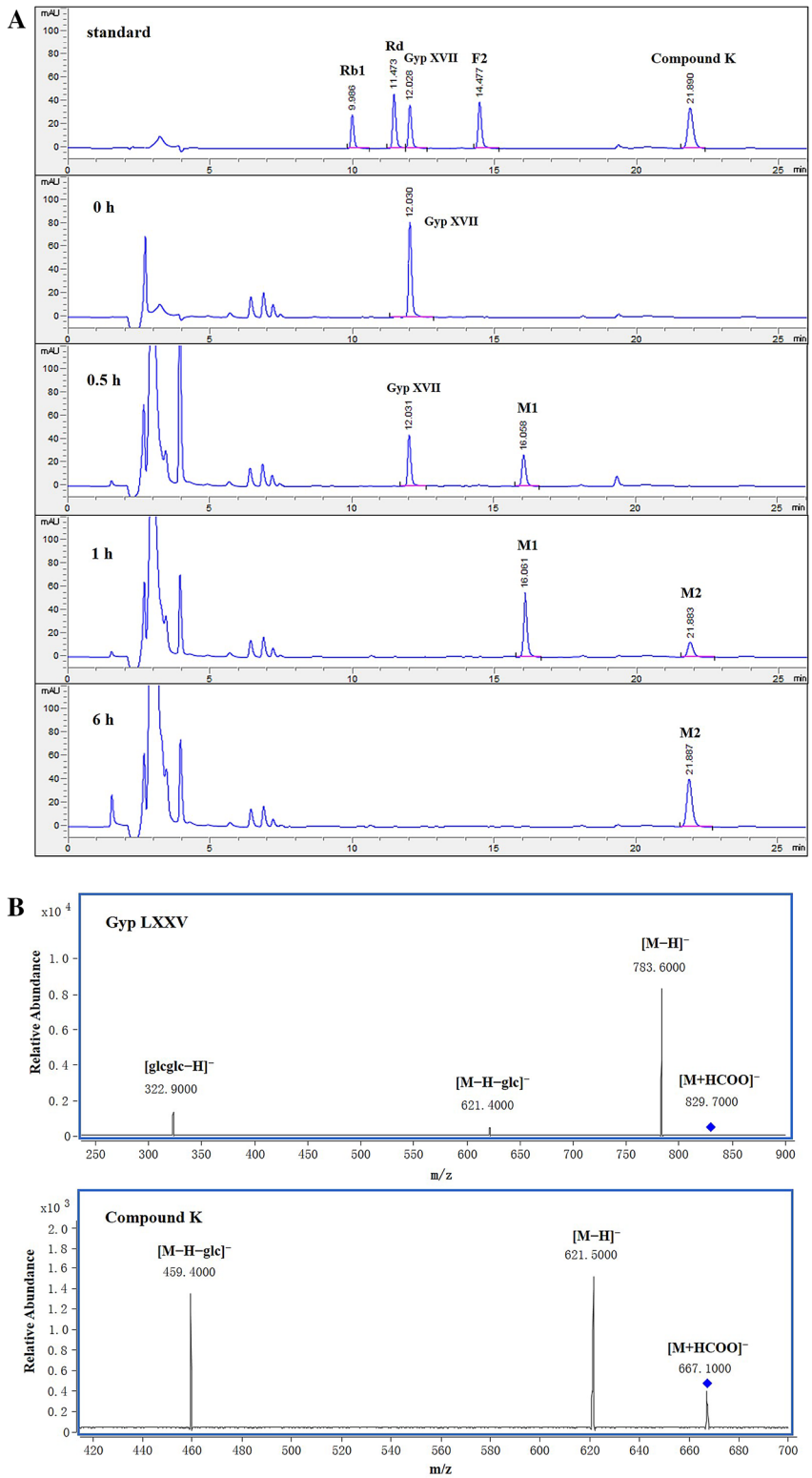

Fig. 3 **a** LC–MS profiles for the conversions of gypenoside XVII to compound K by Bgy1 from *Lactobacillus brevis*. **b** Extracted ion chromatograms (EIC) obtained from the analysis of ginsenosides. *Gyp XVII* gypenoside XVII; *Gyp LXXV* gypenoside LXXV; *M1* metabolites 1; *M2* metabolites 2

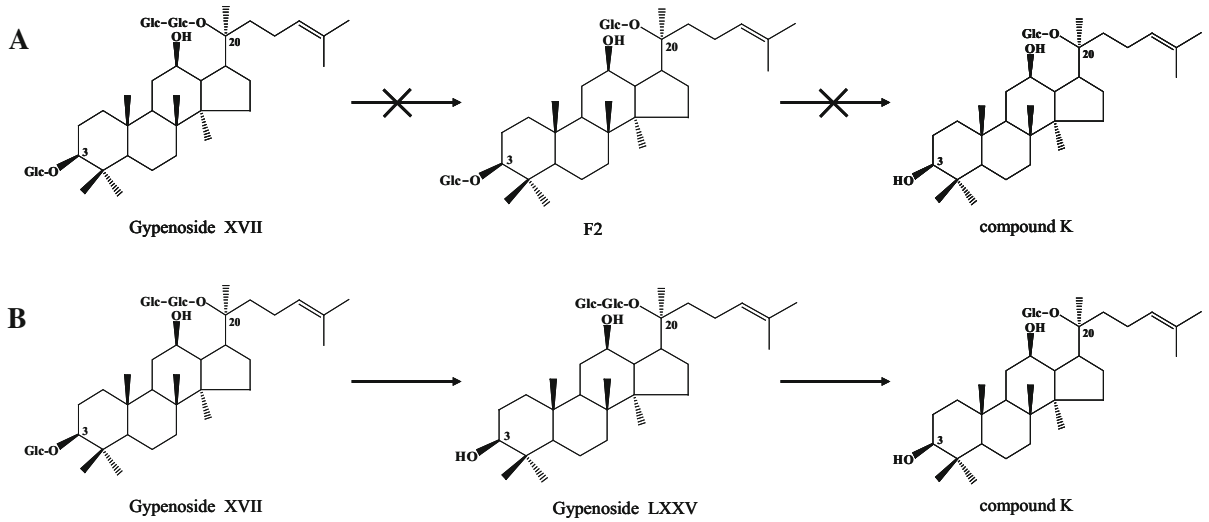

Fig. 4 Transformation pathways of gypenoside XVII by Bgy1 from *Lactobacillus brevis*

Theoretically, there are two pathways for the conversion of gypenoside XVII to compound K. One way is to selectively hydrolyze the C-20 outer sugar moieties to produce ginsenoside F2 and then the C-3 sugar moieties to produce compound K (Fig. 4a). Another way is to selectively hydrolyze the C-3 glucose moieties to produce gypenoside LXXV and then the C-20 outer glucose moieties to produce compound K (Fig. 4b). Therefore, metabolite 1 is ginsenoside F2 or gypenoside LXXV. As the retention time of deglycosylated metabolite 1 (15.9 min) on LC–MS was different from that of ginsenoside F2 (14.2 min), metabolite 1 was identified as gypenoside LXXV (Fig. 3b).

The biotransformations of gypenoside XVII by Bgy1 was quantitatively analyzed by the HPLC LC–MS. Using 0.1 mg enzyme ml^{-1} in 20 mM sodium phosphate buffer at 30 °C and pH 6.0, 1 mg gypenoside XVII ml^{-1} was transformed into 0.58 mg compound K ml^{-1} within 6 h, with a corresponding molar conversion yield of 89 % (Supplementary Fig. 1).

Thus Bgy1 had the hydrolytic pathway of gypenoside XVII → gypenoside LXXV → compound K, first hydrolyzation of the glucose moieties at position C-3 and then the outer glucose moiety of C-20 is hydrolyzed to compound K (Fig. 4b). The ginsenoside-hydrolyzing β-glucosidase from *Sphingobacterium multivorum* GIN723 had the hydrolytic pathway of Rb1 → gypenoside XVII → gypenoside LXXV → compound K → PPD(S) (Kim et al. 2013) through hydrolysis of the outer and inner glucose moieties at position C-3 and hydrolysis of the outer and inner glucose moieties at position C-20 of ginsenoside Rb1, while β-glucosidase from *Actinosynnema mirum* had the hydrolytic pathway of Rb1 → gypenoside XVII → gypenoside LXXV and Rh2(S) → PPD (Cui et al. 2013), through hydrolysis of the outer glucose at position C-3 first and then the hydrolysis of the inner glucose moiety of C-20 to gypenoside LXXV or the removal of the two glucose moieties simultaneously.

In summary, a recombinant ginsenoside-hydrolyzing glycosidase (Bgy1) that belongs to glycosyl hydrolase family 3 was constructed from *L. brevis* for the biotransformation of gypenoside XVII. Bgy1 is able to convert gypenoside XVII into the more pharmacologically active minor ginsenoside, compound K. Therefore, the recombinant Bgy1 is considered potentially useful for the practical preparation of compound K.

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Supporting Information Supplementary Fig. 1—Compound K production from gypenoside XVII by Bgy1 under the optimum conditions.

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