JMB
Journal of Microbiology and Biotechnology

Online ISSN : 1738-8872
Pimt ISSN : 1017-7825

JMB Papers in Press. First Published online Nov 28, 2018
DOI: 10.4014/jmb.1808.08059

Manuscript Number: JMB18-08059

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Article Type: Research article

Keywords: Bifidobacterium breve, biotransformation, compound K, ginsenoside Rd, β-Glucosidase

Title:

Highly Selective Production of Compound K from Ginsenoside Rd by Hydrolyzing Glucose at C-3 Glycoside Using β-glucosidase of *Bifidobacterium breve* ATCC 15700

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Running title: Selective production ginsenoside CK from Rd

Abstract

To investigate a novel β-glucosidase from *Bifidobacterium breve* ATCC 15700 (BbBgl) to produce compound K (CK) via ginsenoside F_2 by highly selective and efficient hydrolysis of the C-3 glycoside from ginsenoside Rd. *BbBgl* gene was cloned and expressed in *E. coli* BL21. The recombinant BbBgl was purified by Ni-NTA magnetic beads to obtain an enzyme with specific activity of 37 U/mg protein using *p*NP-Glc as substrate. The enzyme activity was optimized at pH 5.0, 35°C, 2 or 6 U/ml, and its activity was enhanced by Mn^{2+} significantly. Under the optimal conditions, the half-life of the BbBgl is 180 h, much longer than the characterized β-glycosidases, and the K_m and V_{max} values are 2.7 mM and 39.8 μmol/mg/min for ginsenoside Rd. Moreover, the enzyme exhibits strong tolerance against high substrate concentration (up to 40 g/L ginsenoside Rd) with a molar biotransformation rate of 96% within 12 h. The good enzymatic properties and gram-scale conversion capacity of BbBgl provide an attractive method for large-scale production rare ginsenoside CK using a single enzyme or a combination of enzymes.

Keywords: *Bifidobacterium breve*, biotransformation, compound K, ginsenoside Rd, β-Glucosidase

Introduction

Ginseng (*Panax ginseng* C. A. Meyer) is one of the most famous herbal medicines in Asian. The major family of bioactive constituents of ginseng is ginsenoside. Among the ginsenosides, those dubbed as major ginsenosides such as Rb_1, Rb_2, Rc, Rd, Re, and Rg_1, account for more than 90% (w/w) of the total saponins in ginseng root [1, 2]. Generally, the main constituent of ginseng is ginsenoside Rb_1, followed Re, Rg_1, Rc, Rb_2, and Rd [3]. However, ginseng is a slow-growing plant, difficult to cultivate in the field. Ginsenoside can be obtained only after 4–7 year of cultivation [4]. In recent years, ginseng hairy root culture is widely used in industry for ginsenoside production. In the culture of hairy root, ginsenoside Rd is higher than Rb_1, Rb_2, and Rc [5, 6]. Due to the development of enzyme technology, it is simple to produce ginsenoside Rd using Rb_1, Rb_2, or Rc [7, 8]. In contrast, rare ginsenosides including compound K (CK), F_1, and F_2 are hard to produce because they are low in content or even absent in ginseng or other plants [9]. Nonetheless, most of the minor ginsenosides exhibit much better pharmacological activities than the major counterparts. Ginsenoside CK is the main pharmacologically active metabolites detected in blood after the oral administration of ginsenosides Rb_1, Rb_2, or Rc [10, 11]. With anti-tumor, anti-inflammatory, anticarcinogenic, antidiabetic, anti-allergic, and hepatoprotective activities, CK has attracted wide attention in pharmaceutical application [12, 13]. By means of acid hydrolytic, heating, microbial, and enzymatic transformation techniques, some major ginsenosides could be de-glycosylated at specific position to generate minor ginsenosides such as F_1, F_2, Rh_1, Rg_1, Rg_2, and CK [14, 15]. The conversion of ginsenoside Rd into deglycosylated CK may significantly enhance the biological activity because the latter can function as active compounds and show higher

absorption in the bloodstream [16]. It is hence meaningful to convert the major ginsenosides into the minor ones. Among the various techniques for the preparation of minor ginsenosides, the microorganism or enzyme-based ones are promising for industrial applications because they are highly selective and can be operated under mild conditions with environmental compatibility [17]. Although several studies have shown that Rb_1, Rb_2, Rc, or ginsenoside fraction are used to transform into CK. The biotransformation ability, selectivity, and productivity of most enzymes used for biological conversion do not meet the demands of large-scale and food-grade standards [12, 18]. During biotransformation, it is difficult to produce the sole product CK because there have more intermediates such as Rd, XVII, C-O, C-Y, and F_2 residues [19, 20]. For some β-glycosidases, CK will be further transformed into protopanaxadiol [21]. Furthermore, the application of some β-glycosidases is limited because of its poor activities against Rb_1, Rb_2, and Rc, which possess a disaccharide at C-20 position [7, 14]. The bottleneck of large-scale production CK is largely due to the lack of an available recombinant enzyme that can hydrolyze ginsenoside more efficiently and selectively with a high yield. Therefore, it is very urgent to find ginsenoside-hydrolyzing glycosidase to overcome the difficulties mentioned above.

In the present study, we focus on characterization the recombinant β-glucosidase (BbBgl) originated from food-grade microorganism *Bifidobacterium breve* ATCC 15700 (*B. breve*) and investigation the biotransformation mechanism of Rd into CK by selectively hydrolyze glucose residues of ginsenoside Rd at C-3 position. The good enzymatic properties and gram-scale conversion capacity suggest that the BbBgl could be used for the production of rare ginsenoside CK using a single enzyme or a combination of enzymes in the pharmaceutical

industry.

Materials and methods

Microorganisms, plasmid and biochemical reagents

B. breve, *Escherichia coli* (DH5α), and BL21 (DE3) used in this study were purchased from Beinuo Biotech (Shanghai, China). And the pEASY-Blunt E1 used as an expression vector was purchased from TransGen Biotech (Beijing, China). The ginsenosides purchased from Chengdu Herbpurify (Chengdu, China) were chromatographic grade. All the other reagents were analytical grade.

Construction of expression vector of *BbBgl* gene

The genomic DNA from *B. breve* was extracted as described elsewhere [22] and used as a template for PCR amplification of *BbBgl* gene. The primers were designed based on genomic sequence (GenBank, CP006715.1, 1366947 to 1368227): forward (5′-ATG AGC ATC AAT TGC GCC-3′) and reverse (5′-CTA ATA TTC CCC CGG CAG-3′) primers. To obtain BbBgl with a His-tag at N-terminal, the amplified product was directly ligated to pEASY-Blunt E1 expression vector to produce a recombinant vector. After confirmation by PCR and subsequent sequencing, the recombinant vector was transformed into *E. coli* BL21.

Purification of recombinant BbBgl

The *E. coli* BL21 harboring the *BbBgl* gene was cultured in LB broth containing 100 mg/L ampicillin at 37°C to reach OD_{600}=0.4–0.6, and then induced with a final concentration of 0.5 mM IPTG at 25°C for 8 h. The induced cells were collected by centrifugation at 12,000×*g* for 10 min. And then the pellets were disrupted by sonication in 50 mM citric acid/sodium citrate buffer (pH 5.5) with 1 g/L lysozyme and 5 mg/L DNase. The cell debris

was removed by centrifugation at 12,000×*g* for 15 min. The supernatant containing recombinant BbBgl was incubated with Ni-NTA magnetic agarose beads (Qiagen, Germany) for the collection of the desired protein possessing His-tag. After removing the supernatant by a magnetic separator, the Ni-NTA magnetic agarose beads bound with BbBgl were washed with elution buffer at least twice according to the manufacturer's instructions. The eluates from the beads were collected and concentrated for SDS-PAGE analysis.

Enzyme characterization and determination of kinetic parameters

The purified protein concentration was detected using Folin-phenol reagent. The activity of BbBgl was determined by using *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc) as substrate in 50 mM citric acid/sodium citrate buffer (pH 5.0) at 35°C. The catalytic reaction was ceased by adding 200 mM Na_2CO_3 with volume equal to that of the reaction. The amount of *p*-nitrophenol (*p*NP) released was immediately measured at 405 nm. One unit of the BbBgl activity is defined as the amount of enzyme required to generate 1 μmol *p*NP per minute [22]. To inspect the stability and optimum condition of enzyme activity, the effects of pH and temperature on BbBgl activity were investigated as previously described [22]. The kinetic parameters of BbBgl were measured using *p*NP-Glc, Rd, and F_2 as substrate at concentrations ranging from 0.1 mM to 1 mM. And K_m, K_{cat}, and V_{max} were calculated by fitting the activity data to a linear regression on Lineweaver-Burk double-reciprocal plots. All assays were performed in triplicate.

Analysis of biotransformed products by HPLC

To investigate the ability of recombinant BbBgl for the biotransformation of ginsenosides Rd as well as to study the reaction pathway, we dissolved Rd in methanol and had it incubated

in 50 mM citric acid/sodium citrate buffer (pH 5.0) containing 5 mM Rd and 2 U/ml enzyme at 35°C. The reaction was sampled at regular intervals for a period as shown in the Results and Discussion section and ceased by heating at 80°C for 15 min. The enzymatic products were subsequently extracted with H_2O-saturated n-butanol. After evaporation of solvents, the products were dissolved in methanol and subject to filtration using 0.45 μm microfiltration membranes. HPLC analysis was performed at 203 nm with an Agilent C_{18} column (250 mm×4.6 mm, 5 μm) that was maintained at 30°C. The loading volume of samples was 10 μl and the flow rate was 1 ml/min. The acetonitrile/water (v/v) ratio of eluents was varied from 19:81 to 30:70 for 5 min, from 30:70 to 35:65 for 20 min, and then from 35:65 to 30:70 for 10 min. All HPLC analyses were performed in triplicate.

Results and Discussion

Expression and purification of recombinant BbBgl

The open reading frame (ORF) of *BbBgl* gene was 2,328 bp and the encoded protein was 775-amino acid. The *BbBgl* gene fused to His-tag was expressed using pEASY-Blunt E1 expression system carrying T7 promoter in *E. Coli* BL21 (DE3) followed by the induction of 0.5 mM IPTG at 25°C for 8 h. The BbBgl fused to His-tag was purified using Ni-NTA magnetic agarose beads to obtain protein approximately 80 kDa (Fig. 1), which is similar to the predicted molecular weight according to the amino acid sequences. The purified BbBgl with 37 U/mg showed higher activity than most of β-glycosidases, such as β-glucosidase from *Gordonia terrae* with a specific activity of 16.4 U/mg for Rb_1 [23]. The β-glucosidase of *Thermotoga thermarum* showed high activity of 142 U/mg for *p*NP-Glc and 107 U/mg for Rb_1 respectively, but it need much more amount of enzyme for biotransformation comparing to

147 our experiments [24].

148 **Characterization of recombinant BbBgl**

149 The recombinant BbBgl is active at a temperature range (35–45°C). The optimal
150 temperature for activity is 35°C, and the enzyme is relatively stable between 35 and 40°C.
151 Above 40°C there is drastic decrease of stability (Fig. 2A). The extent of activity loss was
152 32%, 78%, and 91% at 45, 50, and 55°C, respectively. It is apparent that the activity is
153 temperature dependent, in a manner similar to that of *B. breve* glycosidases. For example, the
154 optimal temperature of β-D-xylosidase was 37°C for Ra_1 and Ra_2 to ginsenosides Rb_2 and Rc
155 [25], whereas that of α-L-arabinopyranosidase was 40 and 45°C for Rb_2 and Rc substrates,
156 respectively [26]. The enzymes from human intestinal bacteria and the majority of soil
157 microorganisms for the hydrolysis of ginsenosides are active in the range of 37–45°C, and
158 exhibit hardly any activity above 60°C [27]. It was reported that only the β-D-glucosidase
159 from the thermophilic bacterium shows activity above 50°C [28]. In 50 mM citric
160 acid/sodium citrate buffer, the BbBgl shows optimal activity at pH 5.0, and 91% of maximum
161 activity is still retained at pH 5.5. Above pH 5.5 or below pH 5.0, there is significant decrease
162 of enzyme activity (Fig. 2B). In other words, the pH range for optimal activity of the
163 recombinant BbBgl is similar to that of the other glycosidases isolated from *B. breve* [25, 26].
164 In the present study, the temperature or pH for maximum enzyme activity are the same no
165 matter it is *p*NP-Glc or ginsenoside Rd that is used as substrate. The thermal stability of
166 BbBgl was assayed at 30, 35, 40, and 45°C for different incubation times (Fig. 2C). The
167 thermodynamic parameters show that BbBgl is very stable at 30, 35, 40°C displaying a
168 half-life of 215.8, 180.4, and 171.6 h respectively. The enzyme decreases significantly in

stability above 45°C and the half-life only has 43.8 h. Under the optimal conditions (35°C, pH 5.0), the half-life of BbBgl is quite high comparing with the results found in the literature for other β-glucosidase at its optimized temperature. For the β-glucosidase originated from *Fusarium solani* at 65 °C the half-life is 159 min [29]. The β-glucosidases from *Aureobasidium pullulans* and *Thermoascus aurantiacus* show a half-life of 90 min at 80 °C and about 30 min at 80 °C respectively while the β-glucosidase from *Alteromonas* sp. L82 only has 21 min in half-life at 40 °C [30, 31]. In addition, storage stability is very good since the BbBgl only loss its activity 8-15% after 12 months storage at 4°C (data not shown). A long half-life and appreciable thermostability are desired properties for practical applications.

Kinetic parameters of recombinant BbBgl

Under optimal conditions, the K_m, V_{max}, K_{cat}, and K_{cat}/K_m for reagent-grade *p*NP-Glc, Rd, and F_2 are presented in Table 1. The dependence of substrate concentration followed K_m and V_{max} values of 2.6 mM and 38.7 μmol/mg/min for *p*NP-Glc, 2.7 mM and 39.8 μmol/mg/min for ginsenoside Rd, 2.5 mM and 36.2 μmol/mg/min for F_2. The catalytic efficiencies (K_{cat}/K_m) decreased as *p*NP-Glc (48.8 s^{-1} mM^{-1}), ginsenoside Rd (43.0 s^{-1} mM^{-1}), and F_2 (40.6 s^{-1} mM^{-1}). The catalytic efficiencies for ginsenoside Rd and F_2 is higher than that of β-glucosidase from *Sulfolobus acidocaldarius* for Rb_1 (K_{cat}, 4.8 s^{-1} mM^{-1}), Rc (K_{cat}, 4.5 s^{-1} mM^{-1}), Rd (K_{cat}, 1.0 s^{-1} mM^{-1}), and Rb_2 (K_{cat}, 0.77 s^{-1} mM^{-1}) [28]. The results show that BbBgl is efficient enzyme for hydrolyzing ginsenoside Rd and F_2.

The kinetic property of BbBgl is affected by factors such as temperature, pH, and ionic species. The effects of metal ions on BbBgl activity are shown in Table 2. The enzyme activity is significantly enhanced by Mn^{2+} but obviously inhibited by Cu^{2+} and Hg^{2+}. The

191 presence of Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, or Fe^{2+} does not show significant effect on enzyme
192 activity. The results indicate that the recombinant BbBgl possesses good catalytic activity and
193 environmental compatibility.

194 **Effect of BbBgl activity on the production of CK**

195 The effect of BbBgl activity on the production of CK was investigated at pH 5.0 and 35°C
196 by varying enzyme activity from 0 to 14 U/ml enzyme with 5.0 mM Rd for 12 h. The total
197 conversion of Rd reached 96% using 2 U/ml enzyme, and the yield of F_2 and CK reached 82%
198 and 14% respectively. When 6 U/ml enzyme was used, ginsenoside Rd was biotransformed
199 completely to CK 4.35 mM (2.7 g/L) and F_2 corresponding to 12.4% molar biotransformation
200 rate at 12 h. With increasing enzyme activity, the productivities of F_2 gradually decreased
201 while that of CK increased (Fig. 3A), and when the BbBgl increased to 6 U/ml, ginsenoside
202 Rd was completely converted to CK at 24 h (data not shown).

203 **Effect of substrate concentration on the production of CK**

204 Ginsenoside Rd of 10, 20, 30, 40, 50, and 60 g/L were reacted with purified and crude
205 recombinant BbBgl having 2 U/ml *p*NP-Glc activity at pH 5.0 and 35°C. As shown in Fig. 3B,
206 10, 20, and 30 g ginsenoside Rd/L was completely converted to ginsenoside F_2 and CK within
207 24 h. The biotransformation rate was still as high as 85% within 40 g ginsenoside Rd/L after
208 24 h but decreased obviously at above 50 g ginsenoside Rd/L. To facilitate scaling up of the
209 production, the *E. coli* BL21 harboring *BbBgl* gene was induced by IPTG for 8 h at 25°C
210 using the method mentioned above, and the crude recombinant BbBgl were harvested before
211 purification for ginsenoside Rd biotransformation directly. The recombinant BbBgl in crude
212 protein was soluble form (data not shown) and the activity is similar to the purified ones, the

maximum biotransformation rate reached 89% at 40 g/L ginsenoside Rd at 24 h which is higher than the purified enzyme (Fig. 3C). However, the biotransformation rate of crude enzyme decreased after 24 h, which may be related to the degradation of the BbBgl in the crude protein solution. These results show that the BbBgl has good relative stability and high-substrate tolerance. The biotransformation time of the β-glucosidase from *Acremonium strictum* [32] was 7–8 d, much longer than that in our experiment. Furthermore, the crude enzyme is much cheaper and has good activity. Many scientists have tried to improve the activity, specificity and productivity of enzymes. Quan found that CK was produced from the ginsenoside Rb_1 by the recombinant β-glucosidase from *Microbacterium esteraromaticum*. The recombinant β-glucosidase converted ginsenoside Rb_1 to CK with high productivity using 1 g/L Rb_1 as substrate [33]. An L213A variant of β-glycosidase from *Sulfolobus solfataricus* is screened by using molecular docking and the site-directed mutagenesis which significantly increase the specificity for ginsenoside Rc into CK with molar conversions of 97% containing 4 mM Rc (4.3 g/L) [34]. Although, ginsenoside CK can be produced by using various substrates such as Rb_1, Rb_2, and Rc. As far as we know, this is the largest substrate concentration to produce CK from 40 g/L ginsenoside Rd in a high conversion rate within 24 h. Therefore, 40 g/L ginsenoside Rd can be adopted for the subsequent scaling up of biotransformation.

Substrate specificity and biotransformation pathway

The substrate specificity of recombinant BbBgl is demonstrated under the optimal conditions using *p*NP-Glc, *p*NP-Arap, *p*NP-Araf, *p*NP-Rhap, Rb_1, Rb_2, Rc, Rd, Re, Rg_1, F_2, CK, and two disaccharides (gentiobiose and sophorose) as substrates. As shown in Table 3,

BbBgl exhibit high hydrolytic activity on ginsenoside Rd and F_2, low activity on Rb_1, Rb_2, and Rc, no activity on Re, Rg_1, and CK. The ginsenoside Rb_1, Rb_2, Rc, and Rd, have same protopanaxadiol cores and a disaccharide (glucose-β-(1→2)-glucose) substitution at C-3 site. The main difference among them is the sugar residues substituted at C-20 of aglycone which have three sugar moieties (glucopyranose, arabinopyranose, and arabinofuranose) linking to the glucopyranosyl in β-(1→6) at C-20 of aglycone (Supplementary Fig.1). The results suggest that BbBgl specifically cleave the β-(1→2)-glucosidic linkage at the C-3 position of ginsenoside Rd, Rb_1, Rb_2, and Rc, but do not hydrolyze the β-(1→6)-glucosidic linkage and glucopyranosyl at C-20 of protopanaxadiol-type ginsenosides. It can further hydrolyze the inner glucose moiety attached to the C-3 position. And the specific activity of the enzyme for the ginsenosides followed the order Rd>Rc>Rb_1>Rb_2. BbBgl has no hydrolytic activity for glucopyranose at the C-20, and for rhamnopyranose and glucopyranose at C-6 position in PPT-type ginsenosides. Although the ginsenoside Rb_1, Rb_2, Rc, and Rd have the same glycosidic bond at C-3 site, the recombinant BbBgl is more active towards Rd and has specific stereo preference for C-3 sugars in the hydrolysis of PPD-type ginsenosides. The C-20 position of ginsenosides is a tertiary carbon with large steric structures that inhibits the approach of enzymes, whereas the C-3 position is a secondary carbon with less steric hindrance. And the spatial conformation of disaccharides at C-20 blocked the attack of enzyme to C-3, which resulted in the affinity decreasing between the BbBgl and Rb_1, Rb_2, Rc. As a standard reference, BbBgl exhibit high activity on sophorose (β-(1→2)-glucosidic linkage) compared to gentiobiose (β-(1→6)-glucosidic linkage) (Table 3). Therefore, the BbBgl has high selectivity to β-(1→2)-glucosidic linkage and it hydrolyze the glucoside at the C-3 position in

ginsenosides whereas the enzyme do not hydrolyze the glycoside at the C-6 and C-20 position.

To investigate the transformation mechanism, we performed time-course experiment under the optimal conditions. Ginsenoside F_2 was found on TLC plates after 1 h. There was obvious increase of F_2 after 3 h, and a high level of ginsenoside F_2 was detected at 12 h (Supplementary Fig. 2). Results of similar kind were observed in HPLC analysis. As indicated in Fig. 4, the concentration of F_2 gradually decreased while that of CK increased. Finally, Rd was largely transformed into CK showing a higher CK yield at 24 h. The results demonstrated that the recombinant enzyme is able to biotransform Rd selectively to CK via ginsenoside F_2, having no trace of the unwanted Rd after 24 h. Based on the results, the biotransformation production of CK by consecutive hydrolysis of terminal glucopyranosyl moieties at the C-3 position of ginsenoside Rd follows a pathway of Rd→F_2→CK (Fig. 5). It is noted that the β-glucosidase from *Pyrococcus furiosus*or and *Sulfolobus acidocaldarius* exhibited activities for α-linked arabinopyranose and arabinofuranose moieties such as Rb_2, Rc, and compound Mc [22, 28], and hence are less selectivity specific. In general, the content of ginsenoside Rd is relatively high in some ginseng species and cultured ginseng hairy roots, and ginsenoside Rd is also an important conversion intermediate or end product of Rb_1, Rb_2, and Rc. Therefore, ginsenoside Rd is an important candidate for preparation of CK by using BbBgl based its substrate specificity alone or combination with other glycosidases.

In summary, a member of glycosyl hydrolase family 3, viz. β-glucosidase from *B. breve*, is capable of cleaving glycoside at the C-3 position of ginsenoside Rd to generate deglycosylated ginsenoside CK with high stereo structure specificity. The enzyme with high

stability, long half-life, and high substrate concentration, these properties indicate BbBgl may be an interesting candidate for biotechnological and industrial applications. Our results indicate that it is feasible to develop a specific bioconversion process to obtain rare ginsenoside CK products by the appropriate combination of enzymes and to increase the CK by the overexpression of specific enzymes through genetic engineering.

Acknowledgements

The study was supported by the National Natural Science Foundation of China (81874332, 81673544, 81503452), the Natural Science Foundation of Hunan Province (2017JJ3048, 2016JJ2037), and the Postdoctoral Science Foundation of China (2017T100601, 2016M590746). The critical reading of the manuscript by Prof. C.T. Au, College of Chemistry and Chemical Engineering, Hunan Institute of Engineering is greatly appreciated.

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Table 1 Kinetics parameters of recombinant β-glucosidase originated from *B. breve* for *p*NP-β-D-glucopyranoside and ginsenosides

Substrates	K_m(mM)	K_{cat}(s^{-1})	V_{max}(μmol mg^{-1} min^{-1})	K_{cat}/K_m(s^{-1} mM^{-1})
*p*NP-β-D-glucopyranoside	2.6±0.3	127.1±0.6	38.7±1.8	48.8±0.2
Ginsenoside Rd	2.7±0.7	116.2±0.4	39.8±1.2	43.0±0.4
Ginsenoside F_2	2.5±0.2	101.6±0.7	36.2±1.6	40.6±0.2

393 **Table 2** Effects of metal ions on the activity of recombinant β-glucosidase originated from

394 *B. breve*

Metal ions	Relative activity±SD(%)[a]	
	1 mM	5 mM
Na^{+}	101.3±1.1	95.3±1.1
K^{+}	99.7±0.5	96.1±0.5
Mn^{2+}	115.8±2.1	119.4±2.3
Ca^{2+}	100.1±1.7	94.1±2.1
Mg^{2+}	98.7±0.9	95.1±2.2
Zn^{2+}	103.1±1.9	100.6±1.3
Fe^{2+}	101.3±1.1	96.4±2.6
Cu^{2+}	80.3±1.5	64.3±3.3
Hg^{2+}	20.3±1.7	5.3±1.1
Control	100±2.5	100±3.4

395 [a]Relative activity of the β-glucosidase were assayed using 10 mM *p*NP-Glc as

396 substrate in 50 mM citric acid/sodium citrate buffer (pH 5.0), 2 U/ml enzyme at 35°C

397 for 12 h. The relative activity of *p*NP-Glc was defined as 100%.

398

399 **Table 3** Substrate specificity of recombinant β-glucosidase originated from *B. breve*

Substrates[a]	Relative activity (%)[b]
*p*NP-β-D-glucopyranoside	100±3.1
*p*NP-α-L-arabinopyranoside	5.6±0.6
*p*NP-α-L-arabinofuranoside	0
*p*NP-α-L-rhamnopyranoside	0
Ginsenoside Rb_1	51.4±1.6
Ginsenoside Rb_2	46.2±1.4
Ginsenoside Rc	66.3±2.3
Ginsenoside Rd	94.2±2.9
Ginsenoside Re	0
Ginsenoside Rg_1	0
Ginsenoside F_2	85.3±2.4
Compound K	0
Gentiobiose	0
Sophorose	100±3.5

400 [a]Substrate concentration: 10 mM *p*NP-β-D-glucopyranoside, *p*NP-α-L-arabinopyranoside,
401 *p*NP-α-L-arabinofuranoside, 1 mM Rb_1, Rb_2, Rc, Rd, Re, Rg_1, F2, CK, gentiobiose, and
402 sophorose.
403 [b]The reaction was performed in 50 mM citric acid/sodium citrate buffer (pH 5.0), 2 U/ml
404 enzyme at 35°C for 12 h. The relative activity of pNP-β-D-glucopyranoside was defined as
405 100%.

406 ## Figure legends

407

408 **Fig.1.** SDS-PAGE analysis of β-glucosidase expressed in *E. coli* BL21.

409 M protein molecular weight marker; lanes 1, 2 supernatant of un-induced BL21 cells

410 harboring *BbBgl* gene; and lanes 3, 4 purified β-glucosidase by Ni-NTA magnetic

411 agarose beads.

412

413 **Fig. 2.** Characterization of recombinant β-glucosidase.

414 **(A)** Effect of temperature on the activity of recombinant β-glucosidase determined

415 using *p*NP-Glc as substrate. The activity was assayed in citric acid/sodium citrate

416 buffer (pH 5.0) at 25-55°C for 12 h. **(B)** Effect of pH on the activity of recombinant

417 β-glucosidase determined using *p*NP-Glc as substrate. The activity was assayed at

418 35°C for 12 h in the following buffers (50 mM): citric acid/sodium citrate buffer (pH

419 3.0-5.5) and sodium phosphate buffer (pH 6.0-8.0). **(C)** Thermal stability of

420 recombinant β-glucosidase determined using ginsenoside Rd as substrate. The

421 activity was assayed at 30-45°C in 50 mM citric acid/sodium citrate buffer (pH 5.0).

422 The data represent the means of three separate experiments and error bars represent

423 standard deviation.

424

425 **Fig. 3.** Biotransformation of ginsenosides Rd by recombinant β-glucosidase from *B.*

426 *breve*.

427 **(A)** Effects of β-glucosidase amount on the biotransformation of ginsenoside Rd by

purified recombinant β-glucosidase of *B. breve*. The reaction was performed in 50 mM citric acid/sodium citrate buffer (pH 5.0) containing 5 mM ginsenoside Rd and 0-14 U/ml enzyme at 35°C for 12 h. **(B, C)** Effects of ginsenoside Rd concentration on the production of CK by purified (**B**) and crude (**C**) recombinant β-glucosidase of *B. breve*. The reaction was performed in 50 mM citric acid/sodium citrate buffer (pH 5.0) containing 10-60 g/L ginsenoside Rd and 2 U/ml enzyme at 35°C for 0-120 h. The data represent the means of three experiments and error bars represent standard deviation.

Fig. 4. HPLC profiles for the biotransformation of ginsenoside Rd by recombinant β-glucosidase from *B. breve*.

Ginsenoside Rb_1, Rc, Rd, F_2, Rg_3, and CK were separately used as standards. The reaction was performed in 50 mM citric acid/sodium citrate buffer (pH 5.0) containing 5 mM ginsenoside Rd and 2 U/ml enzyme at 35°C for 0-48 h.

Fig. 5. Proposed biotransformation pathway of ginsenoside Rd to F_2 and CK using β-glucosidase from *B. breve.*

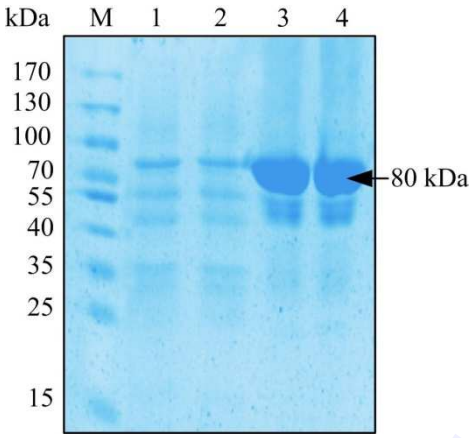

Fig.1

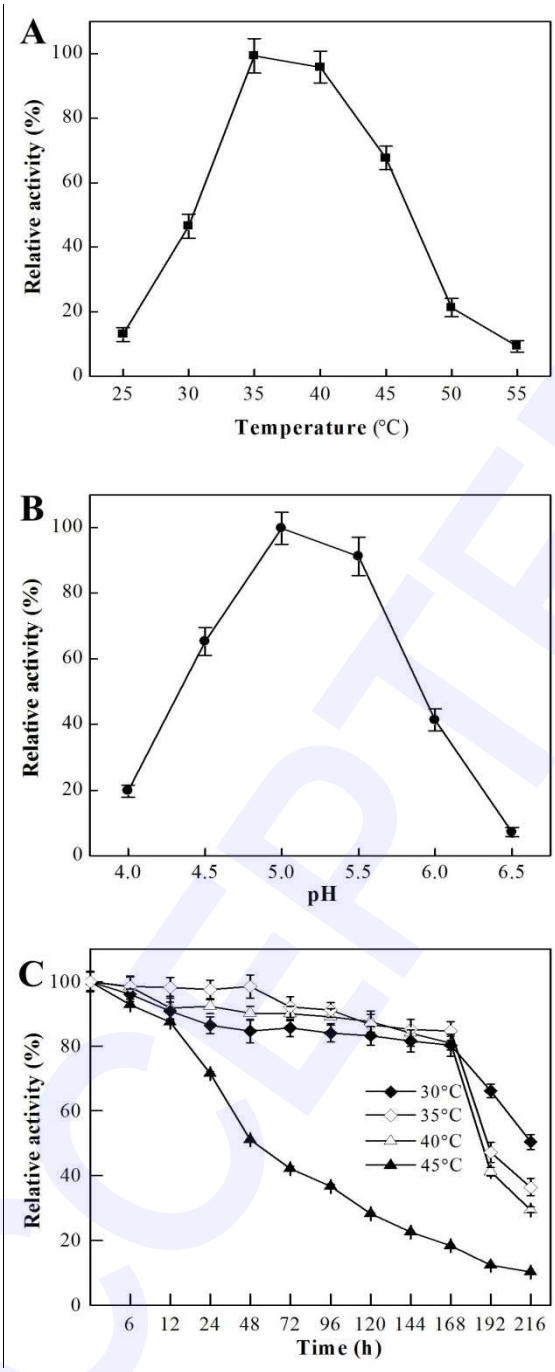

Fig. 2

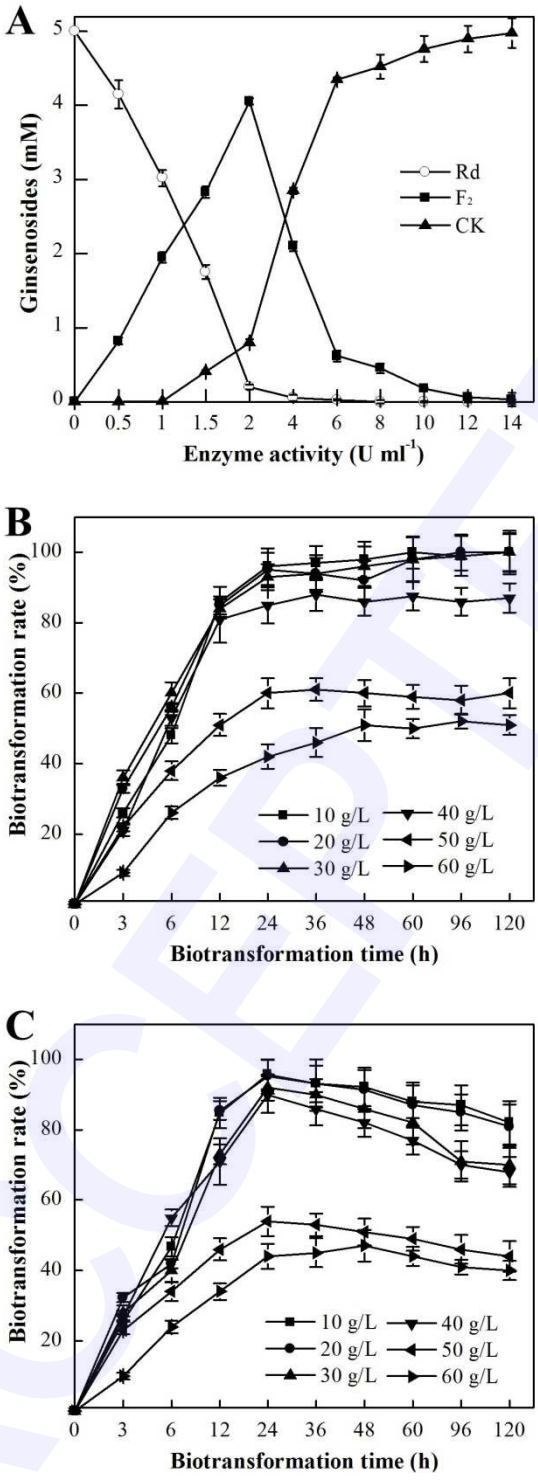

Fig. 3

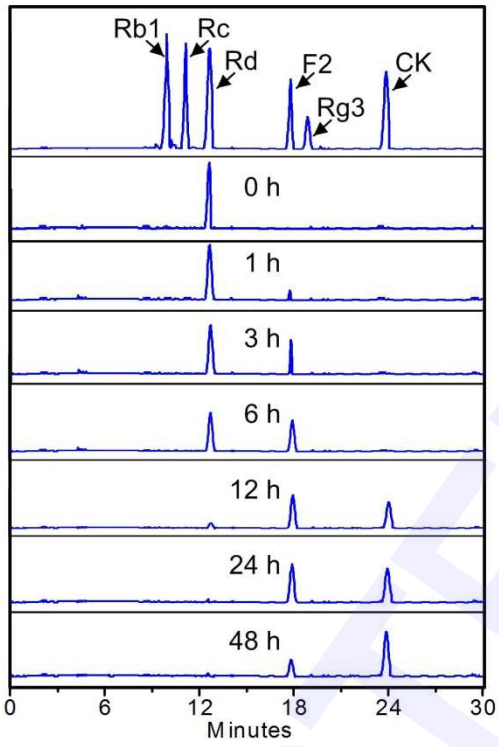

Fig. 4

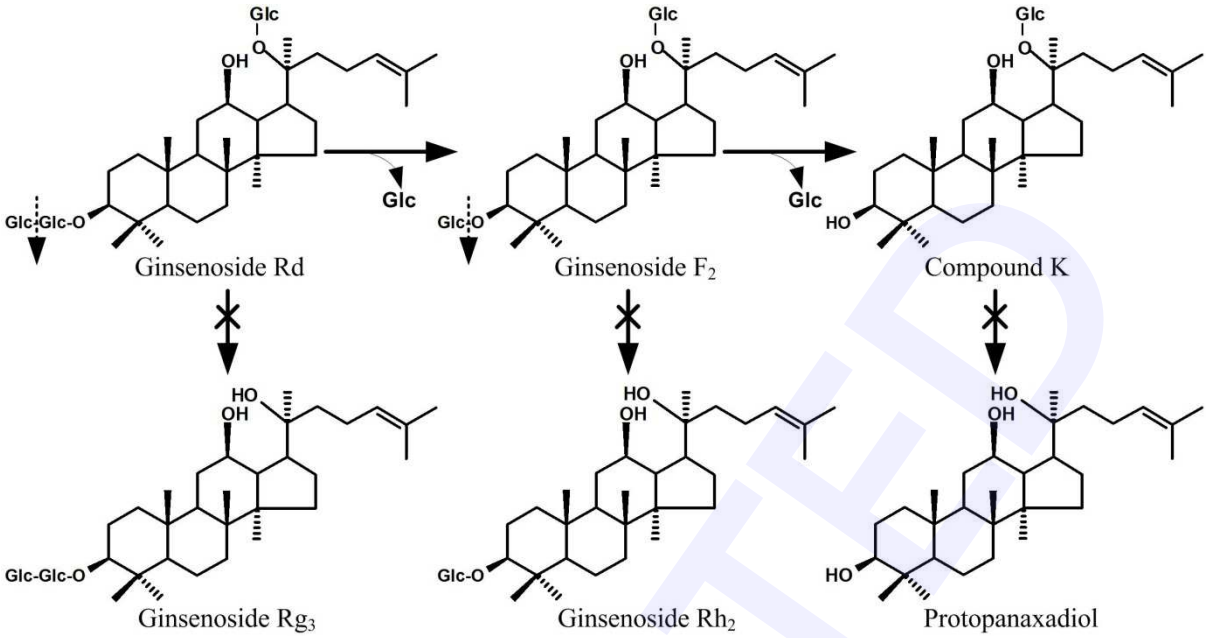

Fig. 5