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

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Received: August 29, 2018
Revised: November 12, 2018
Accepted: November 19, 2018

First published online
November 28, 2018

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^SSupplementary data for this paper are available on-line only at <http://jmb.or.kr>.

pISSN 1017-7825, eISSN 1738-8872

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To investigate a novel β -glucosidase from *Bifidobacterium breve* ATCC 15700 (BbBgl) to produce compound K (CK) via ginsenoside F₂ by highly selective and efficient hydrolysis of the C-3 glycoside from ginsenoside Rd, the *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 pNP-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 Mn²⁺ 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 μ mol/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 of 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 well-known herbal medicines in Asia. The bioactive constituents of ginseng mostly belong to the ginsenoside family. Among the ginsenosides, those dubbed as major ginsenosides, such as Rb₁, Rb₂, Rc, Rd, Re, and Rg₁, account for more than 90% (w/w) of the total saponins in ginseng root [1, 2]. Generally, the main constituent of ginseng is ginsenoside Rb₁, followed Re, Rg₁, Rc, Rb₂, and Rd [3]. However, ginseng is a slow-growing plant that is difficult to cultivate in the field. Ginsenoside can be obtained only after 4–7 years of cultivation [4]. In recent years, ginseng hairy root cultures have become widely used in industry for ginsenoside production. In the culture of hairy root,

ginsenoside Rd is higher than Rb₁, Rb₂, and Rc [5, 6]. Due to the development of enzyme technology, it is simple to produce ginsenoside Rd using Rb₁, Rb₂, or Rc [7, 8]. In contrast, rare ginsenosides including compound K (CK), F₁, and F₂ 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 metabolite detected in blood after the oral administration of ginsenosides Rb₁, Rb₂, or Rc [10, 11]. With anti-tumor, anti-inflammatory, anticarcinogenic, antidiabetic, anti-allergic, and hepatoprotective activities, CK has attracted wide attention as a pharmaceutical application [12, 13]. By means of acid hydrolytic, heating, microbial, and enzymatic

transformation techniques, some major ginsenosides could be de-glycosylated at a 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 an active compound and shows higher absorption in the bloodstream [16]. It is therefore meaningful to convert the major ginsenosides into minor ones. Among the various techniques for the preparation of minor ginsenosides, those that are microorganism or enzyme based are the most promising for industrial applications because they are highly selective and can be operated under mild conditions with environmental compatibility [17]. Several studies however have shown that Rb_1 , Rb_2 , Rc , or ginsenoside fraction can also be transformed 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 CK as a sole product because of 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 of 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 of the recombinant β -glucosidase (BbBgl) originated from the food-grade microorganism *Bifidobacterium breve* ATCC 15700 (*B. breve*) and investigate the biotransformation mechanism of Rd into CK by selectively hydrolyzing 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* (DH5 α), and BL21 (DE3) used in this study were purchased from Beinuo Biotech (China). And the

pEASY-Blunt E1 used as an expression vector was purchased from TransGen Biotech (China). The ginsenosides purchased from Chengdu Herbpurify (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 $\times g$ for 10 min. The pellets were then 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 $\times g$ 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 μ mol *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 ginsenoside 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 H₂O-saturated n-butanol. After evaporation of solvents, the products were dissolved in methanol and subjected to filtration using 0.45 µm microfiltration membranes. HPLC analysis was performed at 203 nm with an Agilent C₁₈ 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 the 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

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

M protein molecular weight marker; lanes 1, 2 supernatant of un-induced BL21 cells harboring *BbBgl* gene; and lanes 3, 4 purified β -glucosidase by Ni-NTA magnetic agarose beads.

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 Rb₁ [23]. The β -glucosidase of *Thermotoga thermarum* showed high activity of 142 U/mg for pNP-Glc and 107 U/mg for Rb₁ respectively, but it needed a much higher amount of enzyme for biotransformation comparing to our experiments [24].

Characterization of Recombinant BbBgl

The recombinant BbBgl is active at a temperature range (35–45°C). The optimal temperature for activity is 35°C, and the enzyme is relatively stable between 35 and 40°C. Above 40°C there is drastic decrease of stability (Fig. 2A). The extent of activity loss was 32%, 78%, and 91% at 45, 50, and 55°C, respectively. It is apparent that the activity is temperature dependent, in a manner similar to that of *B. breve* glycosidases. For example, the optimal temperature of β -D-xylosidase was 37°C for Ra₁ and Ra₂ to ginsenosides Rb₂ and Rc [25], whereas that of α -L-arabinopyranosidase was 40 and 45°C for Rb₂ and Rc substrates, respectively [26]. The enzymes from human intestinal bacteria and the majority of soil microorganisms for the hydrolysis of ginsenosides are active in the range of 37–45°C, and exhibit hardly any activity above 60°C [27]. It was reported that only the β -D-glucosidase from the thermophilic bacterium shows activity above 50°C [28]. In 50 mM citric acid/sodium citrate buffer, the BbBgl shows optimal activity at pH 5.0, and 91% of maximum activity is still retained at pH 5.5. Above pH 5.5 or below pH 5.0, there is significant decrease of enzyme activity (Fig. 2B). In other words, the pH range for optimal activity of the recombinant BbBgl is similar to that of the other glycosidases isolated from *B. breve* [25, 26]. In the present study, the temperature or pH for maximum enzyme activity are the same no matter whether it is pNP-Glc or ginsenoside Rd that is used as substrate. The thermal stability of BbBgl was assayed at 30, 35, 40, and 45°C for different incubation times (Fig. 2C). The thermodynamic parameters show that BbBgl is very stable at 30, 35, 40°C displaying a 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 is only 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 -glucosidases 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.

Fig. 2. Characterization of recombinant β -glucosidase.

(A) Effect of temperature on the activity of recombinant β -glucosidase determined using *p*NP-Glc as substrate. The activity was assayed in citric acid/sodium citrate buffer (pH 5.0) at 25–55°C for 12 h. (B) Effect of pH on the activity of recombinant β -glucosidase determined using *p*NP-Glc as substrate. The activity was assayed at 35°C for 12 h in the following buffers (50 mM): citric acid/sodium citrate buffer (pH 3.0–5.5) and sodium phosphate buffer (pH 6.0–8.0). (C) Thermal stability of recombinant β -glucosidase determined using ginsenoside Rd as substrate. The activity was assayed at 30–45°C in 50 mM citric acid/sodium citrate buffer (pH 5.0). The data represent the means of three separate experiments and error bars represent standard deviation.

Table 1. Kinetic 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} (μ mol mg ⁻¹ min ⁻¹)	K_{cat}/K_m (s ⁻¹ mM ⁻¹)
<i>p</i> NP- β -D-glucopyranoside	2.6 $\pm$ 0.3	127.1 $\pm$ 0.6	38.7 $\pm$ 1.8	48.8 $\pm$ 0.2
Ginsenoside Rd	2.7 $\pm$ 0.7	116.2 $\pm$ 0.4	39.8 $\pm$ 1.2	43.0 $\pm$ 0.4
Ginsenoside F ₂	2.5 $\pm$ 0.2	101.6 $\pm$ 0.7	36.2 $\pm$ 1.6	40.6 $\pm$ 0.2

L82 only has 21 min in half-life at 40°C [30, 31]. In addition, storage stability is very good since the BbBgl only lost 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 F₂ are presented in Table 1. The dependence of substrate concentration followed K_m and V_{max} values of 2.6 mM and 38.7 μ mol/mg/min for *p*NP-Glc, 2.7 mM and 39.8 μ mol/mg/min for ginsenoside Rd, 2.5 mM and 36.2 μ mol/mg/min for F₂, respectively. 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 F₂ (40.6 s⁻¹ mM⁻¹). The catalytic efficiencies for ginsenoside Rd and F₂ are higher than that of β -glucosidase from *Sulfolobus acidocaldarius* for Rb₁ (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 Rb₂ (K_{cat} 0.77 s⁻¹ mM⁻¹) [28]. The results show that BbBgl is an efficient enzyme for hydrolyzing ginsenoside Rd and F₂.

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 Mn²⁺ but obviously inhibited by Cu²⁺ and Hg²⁺. The presence of Na⁺, K⁺, Ca²⁺, Mg²⁺, Zn²⁺, or Fe²⁺ does not show significant effect on enzyme activity. The results indicate that the recombinant BbBgl possesses good catalytic activity and environmental compatibility.

Effect of BbBgl Activity on the Production of CK

The effect of BbBgl activity on the production of CK was investigated at pH 5.0 and 35°C by varying enzyme activity from 0 to 14 U/ml enzyme with 5.0 mM Rd for 12 h. The total conversion of Rd reached 96% using 2 U/ml enzyme, and the yield of F₂ and CK reached 82% and 14% respectively. When 6 U/ml enzyme was used, ginsenoside

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

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

^aRelative activity of the β -glucosidase was assayed using 10 mM *p*NP-Glc as substrate in 50 mM citric acid/sodium citrate buffer (pH 5.0), 2 U/ml enzyme at 35°C for 12 h. The relative activity of *p*NP-Glc was defined as 100%.

Rd was biotransformed completely to CK 4.35 mM (2.7 g/l) and F₂ corresponding to 12.4% molar biotransformation rate at 12 h. With increasing enzyme activity, the productivities of F₂ gradually decreased while that of CK increased (Fig. 3A), and when the BbBgl increased to 6 U/ml, ginsenoside Rd was completely converted to CK at 24 h (data not shown).

Effect of Substrate Concentration on the Production of CK

Ginsenoside Rd of 10, 20, 30, 40, 50, and 60 g/l were reacted with purified and crude recombinant BbBgl having 2 U/ml *p*NP-Glc activity at pH 5.0 and 35°C. As shown in Fig. 3B, 10, 20, and 30 g of ginsenoside Rd/L were completely converted to ginsenoside F₂ and CK within 24 h. The biotransformation rate was still as high as 85% within 40 g ginsenoside Rd/L after 24 h but decreased obviously at above 50 g of ginsenoside Rd/L. To facilitate scaling up of the production, the *E. coli* BL21 harboring *BbBgl* gene was induced by IPTG for 8 h at 25°C using the method mentioned above, and the crude recombinant BbBgl was harvested before purification for ginsenoside Rd biotransformation directly. The recombinant BbBgl in crude protein was soluble form (data not shown) and the activity is similar to the purified ones, while 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.

Fig. 3. Biotransformation of ginsenoside Rd by recombinant β -glucosidase from *B. breve*. (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.

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 Rb₁ by the recombinant β -glucosidase from *Microbacterium esteraromaticum*. The recombinant β -glucosidase converted ginsenoside Rb₁ to CK with high productivity using 1 g/l Rb₁ as substrate [33]. An L213A variant of β -glycosidase from *Sulfolobus solfataricus* was screened by using molecular docking and the site-directed mutagenesis significantly increased 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 Rb₁, Rb₂, 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 pNP-Glc, pNP-Arap, pNP-Araf, pNP-Rhap, Rb₁, Rb₂, Rc, Rd, Re, Rg₁, F₂, CK, and two disaccharides (gentiobiose and sophorose) as substrates. As shown in Table 3, BbBgl exhibits high hydrolytic activity on ginsenoside Rd and F₂, low activity on Rb₁, Rb₂, and Rc, no activity on Re, Rg₁, and CK. The ginsenosides Rb₁, Rb₂, Rc, and Rd, have the same protopanaxadiol cores and a disaccharide (glucose- β -(1 $\rightarrow$ 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 $\rightarrow$ 6) at C-20 of aglycone (Fig. S1). The results suggest that BbBgl specifically cleaves the β -(1 $\rightarrow$ 2)-glucosidic linkage at the C-3 position of ginsenosides Rd, Rb₁, Rb₂, and Rc, but does not hydrolyze the β -(1 $\rightarrow$ 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>Rb₁>Rb₂. BbBgl has no hydrolytic activity for glucopyranose at the C-20, or for rhamnopyranose and glucopyranose at C-6 position in

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

Substrates ^a	Relative activity (%) ^b
pNP- β -D-glucopyranoside	100 $\pm$ 3.1
pNP- α -L-arabinopyranoside	5.6 $\pm$ 0.6
pNP- α -L-arabinofuranoside	0
pNP- α -L-rhamnopyranoside	0
Ginsenoside Rb ₁	51.4 $\pm$ 1.6
Ginsenoside Rb ₂	46.2 $\pm$ 1.4
Ginsenoside Rc	66.3 $\pm$ 2.3
Ginsenoside Rd	94.2 $\pm$ 2.9
Ginsenoside Re	0
Ginsenoside Rg ₁	0
Ginsenoside F ₂	85.3 $\pm$ 2.4
Compound K	0
Gentiobiose	0
Sophorose	100 $\pm$ 3.5

^aSubstrate concentration: 10 mM pNP- β -D-glucopyranoside, pNP- α -L-arabinopyranoside, pNP- α -L-arabinofuranoside, 1 mM Rb₁, Rb₂, Rc, Rd, Re, Rg₁, F₂, CK, gentiobiose, and sophorose.

^bThe reaction was performed in 50 mM citric acid/sodium citrate buffer (pH 5.0), 2 U/ml enzyme at 35°C for 12 h. The relative activity of pNP- β -D-glucopyranoside was defined as 100%.

PPT-type ginsenosides. Although ginsenosides Rb₁, Rb₂, 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 Rb₁, Rb₂, Rc. As a standard reference, BbBgl exhibits high activity on sophorose (β -(1 $\rightarrow$ 2)-glucosidic linkage) compared to gentiobiose (β -(1 $\rightarrow$ 6)-glucosidic linkage) (Table 3). Therefore, BbBgl has high selectivity to β -(1 $\rightarrow$ 2)-glucosidic linkage and it hydrolyzes the glucoside at the C-3 position in ginsenosides whereas the enzyme does not hydrolyze the glycoside at the C-6 and C-20 position.

To investigate the transformation mechanism, we performed a time-course experiment under the optimal conditions. Ginsenoside F₂ was found on TLC plates after 1 h. There was obvious increase of F₂ after 3 h, and a high level of ginsenoside F₂ was detected at 12 h (Fig. S2).

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

Ginsenosides Rb₁, Rc, Rd, F₂, Rg₃, 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.

Results of similar kind were observed in HPLC analysis. As indicated in Fig. 4, the concentration of F₂ 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 F₂, 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 $\rightarrow$ F₂ $\rightarrow$ CK (Fig. 5). It is noted that the β -glucosidase from *Pyrococcus furiosus* and *Sulfolobus acidocaldarius* exhibited activities for α -linked arabinopyranose and arabinofuranose moieties such as Rb₂, 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 Rb₁, Rb₂, and Rc. Therefore, ginsenoside Rd is an important candidate for preparation of CK by using BbBgl based on its substrate specificity alone or in 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. As an 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.

Fig. 5. Proposed biotransformation pathway of ginsenoside Rd to F₂ and CK using β -glucosidase from *B. breve*.

Acknowledgments

The study was supported by the National Natural Science Foundation of China (81874332, 81673544, 81503452), the Natural Science Foundation of Hunan Province (2017JJ3048, 2016JJ2037), the National Training Programs of Innovation and Entrepreneurship for Undergraduates (201811342007) 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.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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