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Author: Ye Yuan Yanbo Hu Chenxing Hu Jiayi Leng Honglei Chen Xuesong Zhao Juan Gao Yifa Zhou

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1 Overexpression and characterization of a glycoside hydrolase family 1 enzyme from

2 *Cellulosimicrobium cellulans* sp.21 and its application for minor ginsenosides production

3

4 Ye Yuan[1], Yanbo Hu[1], Chenxing Hu[1], Jiayi Leng[1], Honglei Chen[1], Xuesong Zhao[2], Juan Gao[1,*],

5 Yifa Zhou[1,*]

6

7 [1]*Jilin Province Key Laboratory on Chemistry and Biology of Natural Drugs in Changbai*

8 *Mountain, School of Life Sciences, Northeast Normal University, Changchun 130024, PR China*

9 [2]*College of Mining Industry, Liaoning Technical University, Fuxin 123000, PR China*

10

11 [*]Corresponding author, Juan Gao and Yifa Zhou. Jilin Province Key Laboratory on Chemistry and

12 Biology of Natural Drugs in Changbai Mountain, School of Life Sciences, Northeast Normal

13 University, Changchun 130024, PR China.

14 Tel/Fax: +86-431-85098212; E-mail: gaoj199@nenu.edu.cn (Juan Gao); zhouyf383@nenu.edu.cn

15 (Yifa Zhou).

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1 **Abstract**

2 A novel β-glucosidase gene (*ccbgl1a*) was cloned from the ginsenosides-transforming strain
3 *Cellulosimicrobium cellulans* sp.21. This enzyme was overexpressed in *Escherichia coli*, the
4 recombinant β-glucosidase (CcBgl1A) containing N-terminal His-tag was sufficiently purified by
5 nickel metal affinity chromatography with purification factor of 1.9-fold and specific activity of
6 31.5 U/mg**.** The molecular mass of recombinant CcBgl1A was estimated to be approximately 46
7 kDa. CcBgl1A exhibited optimal activity at 35°C and pH 5.5. However, above 40°C, the enzyme
8 stability significantly decreased. The enzyme showed high bioconversion ability on
9 protopanaxadiol-type ginsenosides mixture (PPDGM), which could hydrolyze the outer C-3
10 glucose moieties of ginsenosides Rb1, Rb2, Rc and Rd into the rare ginsenosides Gypenoside
11 XVII (Gyp XVII), compound O, ginsenoside Mb and ginsenoside F2. Scaled-up production using
12 1 g of the PPDGM resulted in 292 mg Gyp XVII, 134 mg CO, 184 mg Mb, and 62 mg F2, with
13 chromatographic purities. These results suggest that CcBgl1A would be potentially useful in the
14 preparation of pharmacologically active minor ginsenosides Gyp XVII, CO, Mb and F2.

15 **Keywords:** Biotransformation; *Cellulosimicrobium cellulans* sp.21; ginsenosides; β-glucosidase;
16 overexpression

17

18 **1. Introduction**

19 Ginseng, the root of *Panax ginseng* C.A. Meyer, has been used as a traditional medicine in
20 Asian counties to strengthen immunity, supply nutrition and decrease fatigue for over 2000 years
21 [1-3]. Ginsenosides are reported to be the major active components of ginseng [4, 5]. The major
22 ginsenosides Rb1, Rb2, Rc, Rd, Re, and Rg1 represents more than 80% of total ginseng

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1 ginsenosides [6, 7]. Some ginsenosides, such as Gypenoside XVII (Gyp XVII or GXVII), F2,
2 Compound O (CO) and Mb, that are deglycosylated from the major ginsenosides, exist in smaller
3 amounts or are absent in ginseng. Deglycosylated ginsenosides are more pharmaceutically active
4 than major glycosylated ginsenosides, because of their small size, high bioavailability and good
5 permeability across the cell membrane [8, 9]. Thus, the minor ginsenosides have attracted more
6 attention in recent years. These minor ginsenosides are difficult to obtain from a direct extraction
7 of ginseng. However, the minor ginsenosides Gyp XVII, CO, Mb and F2 can be produced by
8 hydrolyzing the β-(1→2)-glucosidic linkage at the C-3 position of the major ginsenosides Rb1,
9 Rb2, Rc and Rd, respectively. Many methods including heating, acidic and alkali treatments, and
10 enzymatic conversion, have been developed for the production of the minor ginsenosides [10-12].
11 Among these, the enzymatic conversion is the most used because of its high specificity, yield, and
12 productivity [13-16].

13 Some bacteria and fungi, and the ginsenoside-hydrolyzing enzymes produced by
14 microorganisms are reported for ginsenoside conversion [17, 18]. Compared to native
15 ginsenoside-hydrolyzing enzymes or gensinosidase producing microorganisms, recombinant
16 enzyme are better in ginsenoside biotransformation due to its high yield, easy preparation
17 procedure and more stable biotransformation process [9]. Several reports focused on
18 biotransformation of ginsenosides by recombinant enzymes [9, 14, 16, 19-23]. In the present study,
19 a ginsenoside-hydrolyzing β-glucosidase gene *ccbgl1a* was cloned from *Cellulosimicrobium*
20 *cellulans* sp. 21, the recombinant protein, CcBgl1A, was overexpressed, purified and the
21 enzymatic properties were investigated. The biotransformation of the major ginsenosides Rb1,
22 Rb2, Rc, and Rd by CcBgl1A, to the rare ginsenosides Gyp XVII, compound O, compound Mb,

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1 and F2 was also investigated.

2

3 **2. Materials and Methods**

4 *2.1 Chemicals and Reagents*

5 Substrates *p*-nitrophenyl (*p*NP)-β-glucopyranoside (*p*NPβGlc), *p*NP-α-glucopyranoside
6 (*p*NPαGlc), *p*NP-β-galactopyranoside (*p*NPβGal), *p*NP-α-galactopyranoside (*p*NPαGal),
7 *p*NP-β-mannopyranoside (*p*NPβMan), *p*NP-α-mannopyranoside (*p*NPαMan),
8 *p*NP-β-xylopyranoside (*p*NPβXyl), *p*NP-α-L-arabinofuranoside (*p*NPαAra*f*), 4-methylumbelliferyl
9 β-D-glucopyranoside (MUG) were purchased from Sigma (St. Louis, MO, USA). All other
10 chemicals and reagents were of analytical grade. Protopanaxadiol-type ginsenosides mixture
11 (PPDGM), single ginsenosides Rb1, Rb2, Rc, Rd, F2, Gyp XVII, compound O and compound Mb,
12 were prepared in our lab as described before [12].

13 *2.2 Bacterial Strains, Plasmids and Media*

14 *C. cellulans* sp. 21 with the collection number CGMCC 7587 in China General Microbiological
15 Culture Collection Center was grown on Luria Bertani (LB) agar at 30°C for 24 h. The stain was
16 identified by 16S rDNA sequencing. The 16S rDNA amplification was performed using 27F
17 (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGCTACCTTGTTACGACTT) as primers.
18 The program for PCR was 94°C for 5 min, 30 cycles of three steps (94°C for 1 min, 55°C for 30 s,
19 and 72°C for 1 min), and followed by 72°C for 10 min additionally. Obtained 1409-bp amplicon
20 was analyzed by Sangon Biotech. Co. (Shanghai, China) with 27F and 1492R as sequencing
21 primers.

22 *E. coli* BL21 (DE3) and pET-28a (+) (Novagen, Madison, WI, USA) were used as host and

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1 expression vector sources, respectively. Recombinant *E. coli* strains were grown in LB broth with
2 50 μg/ml kanamycin at 37°C.

3 *2.3 Basic Recombinant DNA Methods*

4 Genomic DNA of *C. cellulans* sp. 21 was isolated using DNeasy Tissue kit (Qiagen, Hilden,
5 Germany). Primers *ccbgl1a*-F (5'-GGAATTCCATATGGTGTCGATCACCTTCCCCGAGTC-3')
6 and *ccbgl1a*-R (5'-CGGGATCCTTATCAGTCGTCGAGCGAGACGGTC-3') were used for gene
7 *ccbgl1a* amplification to introduce *Nde*I and *Bam*HI restriction sites (underlined) for cloning to the
8 pET-28a (+). PCR was performed using Phusion polymerase and the following program: 98°C for
9 30 s, 30 successive cycles of 98°C for 10 s, 68°C for 45 s, and 72°C for 1 min, and final 72°C for
10 10 min. The PCR product and pET28a (+) were digested with *Nde*I and *Bam*HI and ligated to
11 insert the *ccbgl1a* gene into pET28a (+) for expression. The resulting recombinant
12 pET28a-*ccbgl1a* was used to transform *E. coli* BL21 (DE3) cells. The positive transformants were
13 verified by DNA sequencing. All enzymes used were from New England Biolab (Beverly, MA).
14 The DNA isolations, restriction enzyme digestions, ligations and transformations were performed
15 as the suppliers' recommendations.

16 *2.4 Expression and Purification of Enzyme CcBgl1A*

17 Recombinant *E. coli* BL21 (DE3) harboring the recombinant plasmid was grown in 200 mL of
18 LB broth with 50 μg/ml kanamycin at 37°C. When the OD 600_{nm} reached 0.5, the culture was
19 induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and then grown for another 20
20 h at 25°C. Subsequently, the cells were harvested and disrupted by sonication in binding buffer (10
21 mM imidazole, 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5). Cell debris were removed by
22 centrifugation (14,000 × g, 4°C, 30 min) to obtain the crude extract.

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1 Protein CcBgl1A carrying His-tag was loaded onto Ni sepharose fastflow column (GE
2 healthcare). The column of 5 mL was pre-equilibrated by the binding buffer, washed with 20 mL
3 washing buffer (20 mM imidazole, 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5) and then eluted with 20
4 mL eluting buffer (250 mM imidazole, 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5). The active
5 fractions were pooled, desalted by membrane ultrafiltration with Amicon YM10 centriprep device
6 (Millipore, Bedford, Massachusetts, USA) and used for further characterizations.
7 The purified CcBgl1A was analyzed by SDS-PAGE on 10% separating gel. Densitometry of the
8 stained gels was used to estimate the quantity of enzyme in the preparation. Protein concentrations
9 were determined by Bradford method using bovine serum albumin (BSA) as a standard. For
10 detection of in-gel β-glucosidase activity, the purified CcBgl1A was analyzed by native PAGE
11 using 8% separation gel under non-reducing conditions. After electrophoresis, the gel was washed
12 twice with 50 mM sodium acetate buffer (pH 5.5) for 10 min and then overlaid with 2 mM MUG
13 in 50 mM sodium acetate buffer (pH 5.5) at 25°C for 5 min. The fluorescent band was visualized
14 under UV light and proteins were stained with Coomassie brilliant blue G-250.
15 *2.5 Enzymatic Activity Analysis of CcBgl1A on Different Substrates*
16 Enzymatic activities of CcBgl1A on various substrates including *p*NP-linked glycosides,
17 disaccharides and ginsenosides were assayed. The reaction mixture of 200 μl composed of 50 mM
18 sodium acetate buffer (pH 6.0), 5 mM substrate and 2 μg β-glucosidase, was incubated at 30°C for
19 10 min. The reaction mixture without enzyme was used as a blank. The reaction was stopped by
20 the addition of 1 M NaOH (100 μl). For *p*NP-linked glycosides, the absorbance of the mixture was
21 measured at 405 nm by BioTek ELx808 microplate reader (Winooski, VT, USA). The absorbance
22 was compared to a standard curve of *p*-nitrophenol in a solution of the same composition (0.1-5

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1 mM). The enzymatic activities of CcBgl1A on sophorose, laminaribiose, cellobiose and
2 gentiobiose were measured with dinitrosalicylic acid reagent at 520 nm by determining the
3 reducing sugars, with glucose as a standard (0.1-5 mM) [24]. The hydrolysis activities on
4 ginsenosides by CcBgl1A were determined by HPLC (see analytical methods). The amount of
5 product was calculated according to its peak area. The standard curve of Gyp XVII, CO, Mb and
6 F2 was used at a concentration of 0.1-5 mM. One unit (U) of β-glucosidase activity was defined as
7 the amount of enzyme releasing 1 μmol/min of *p*-nitrophenyl/reducing sugar/ginsenoside under
8 assay conditions.

9 *2.6 Effect of pH and Temperature on Enzyme Activity*

10 The dependence of the purified CcBgl1A on pH was determined in different buffers (50 mM
11 final concentration) at different pH (pH 2.0-6.0, sodium acetate buffer; pH 6.0-8.0,
12 Na_2HPO_4-NaH_2PO_4 buffer; pH 8.0-11.0, Glycine-NaOH buffer) using *p*NPβGlc as a substrate.
13 The pH stability was investigated under standard assay conditions after incubation of the purified
14 enzyme for 24 h at 4°C in the buffer without substrate. The optimum temperature was determined
15 by measuring enzymatic activity at pH 5.5 in the temperature range of 20-80°C. Temperature
16 stability was measured by analyzing residual activity after incubation of aliquots of enzyme at
17 different time and different temperatures.

18 *2.7 Effect of Metal Ions and Chemical Reagents on Enzyme Activity*

19 The effects of metals and other chemicals on CcBgl1A activity were determined. CcBgl1A
20 activity was tested in the presence of 5 or 50 mM (final concentration) of metals or other
21 chemicals for 1 h at 30°C. The remaining activity was determined using *p*NPβGlc as a substrate
22 (final concentration 5 mM) as described before, and activities are expressed as a percentage of the

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1 activity obtained in the absence of the compound.

2 *2.8 Determination of Kinetic Parameters*

3 To determine the kinetic parameters on various substrates, substrate concentration was varied
4 (0.05 mM – 2 mM) and reaction time of 15 min was chosen to ensure initial rates. Buffer and
5 enzyme concentrations used are identical to that stated above. The K_m and V_{max} values were
6 calculated from GraphPad Prism V5.

7 *2.9 Scaled-up Production of Gyp XVII, CO, Mb and F2 by Crude Recombinant CcBgl1A*

8 Scaled-up production of minor ginsenosides Gyp XVII, CO, Mb and F2 was performed in a 1-L
9 stirred tank reactor. 200-ml reaction mixture contained 5 mg/ml of substrate PPDGM and crude
10 recombinant CcBgl1A (final protein concentration 1 mg/ml) in 50 mM phosphate buffer (pH 5.5).
11 After reaction at 30 °C for 2 h at 100 rpm, the mixture was extracted with one volume of n-butanol
12 twice. The n-butanol extracts were pooled and evaporated *in vacuo*, then applied onto preparative
13 Shim-pack PREP-ODS (H) column (20 mm × 250 mm, 5 μm) connected to an HPLC system
14 (Shimadzu, Japan). The column was eluted at the flow rate of 5.0 ml/min with the following
15 gradient program: 0–10 min, 32% acetonitrile (in distilled water, v/v); 10–40 min, 32%–60%
16 acetonitrile; and 40–50 min, 60% acetonitrile, monitored by the absorbance at 203 nm. Fractions
17 were taken every 5-ml elution. Each product was pooled and evaporated *in vacuo*.

18 *2.10 Analytical Methods*

19 TLC was carried out using a silica gel G60 plate and a developing solvent consisting of
20 chloroform, methanol and water (65:35:10, v/v/v, lower phase). The ginsenosides developed on
21 the plate were stained by spraying with 5% sulphuric acid in ethanol (v/v), and then heating at
22 110°C for 5 min. HPLC analysis was performed using analytical Shim-pack PREP-ODS (H)

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1 column (4.6 mm × 250 mm, 5 μm) connected to an HPLC system (Shimadzu, Japan), eluted at the
2 flow rate of 1.0 ml/min with the following gradient program: 0–10 min, 32% acetonitrile (in
3 distilled water, v/v); 10–40 min, 32%–60% acetonitrile; and 40–50 min, 60% acetonitrile,
4 monitored by the absorbance at 203 nm. ^{13}C-NMR spectra were carried out on a Bruker Av 600
5 NMR spectrometer at 150 MHz with CD_3OD as the solvent and TMS as the internal standard.
6 *2.11 Nucleotide Sequence Accession Numbers*
7 The sequences for the 16S rRNA and CcBgl1A genes from *C. cellulans* sp. 21 were deposited in
8 GenBank under accession numbers KR349463 and KR185312, respectively.
9
10 **3. Results and Discussion**
11 *3.1 Gene Cloning and Analysis of CcBgl1A Gene*
12 Biotransformation is considered to be a desirable method for the preparation of bioactive minor
13 ginsenosides because of its high specificity, yield, and productivity. Previously, we isolated a
14 ginsenoside-hydrolyzing strain from soil, which was identified by 16S rDNA sequencing to be *C.*
15 *cellulans*. In the present study, a ginsenoside-hydrolyzing β-glucosidase gene belonging to GH
16 family 1 was cloned from the genomic DNA of *C. cellulans* sp. 21. Although some
17 ginsenoside-hydrolyzing β-glucosidases from bacteria and fungi have been studied extensively [9,
18 11, 14, 19-21, 23], this represents the first report on the overexpression and characterization of *C.*
19 *cellulans* ginsenoside-hydrolyzing enzyme.
20 The β-glucosidase gene consisting of 1,173 bp encoding 390 amino acids with a theoretical
21 molecular mass of 43.33 kDa and a theoretical *p*I value of 4.85
22 (http://web.expasy.org/compute_pi/). Signal sequence of CcBgl1A was analyzed by the SignalP4.1

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1 server (http://www.cbs.dtu.dk/services/SignalP/), the lack of a signal sequence suggests its
2 intracellular location in *C. cellulans*. The multiple amino acid sequence alignment indicated
3 CcBgl1A exhibited identities with some characterized GH1 ginsenoside-hydrolyzing enzymes (Fig.
4 S1), such as β-glucosidases from *Arthrobacter chlorophenolicus* (43%), *Sphingopyxis alaskensis*
5 (33%), *Sphingomonas* sp. 2F2 (30%), *Pyrococcus furiosus* (29%), and β-galactosidase from
6 *Sulfolobus acidocaldarius* (25%) [9, 14, 19-20, 23]. Amino acid sequence comparisons revealed
7 that CcBgl1A shared the same conserved catalytic residue sequences with other GH1
8 ginsenoside-hydrolyzing enzymes, the Glu158 in conserved region Asn-Glu-Pro (NEP) was
9 identified as the acid/base catalyst, and Glu304 in conserved region Glu-Asn-Gly (ENG) was
10 identified as the catalytically nucleophile.

11 *3.2 Overexpression, Purification, and Molecular Mass Determination of CcBgl1A from C.*
12 *cellulans sp. 21*

13 The recombinant enzyme was purified as a soluble protein from crude *E. coli* extracts. In order
14 to maximize the yield of the fusion protein in a soluble form, different induction conditions were
15 tested. It was found that 0.5 mM IPTG at 25°C for 20 h cultivation after induction produced the
16 maximum amount of soluble active fusion enzyme (data not shown). Under the optimal induction
17 condition, the concentration of total protein was 0.5 g/l culture and the specific activity of
18 recombinant β-glucosidase was 8350 U/l culture. This high productivity makes the recombinant
19 CcBgl1A possible for industrial application.

20 The fusion protein was purified by Ni sepharose fastflow column, β-glucosidase from *C.*
21 *cellulans* sp. 21 was purified with a final purification of 1.9-fold, with a yield of 72%, and a
22 specific activity of 31.5 U/mg (Table 1). The expressed protein analyzed by SDS-PAGE gel

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1 showed a single band with a molecular mass of approximately 46 kDa (Fig. 1a), consistent with
2 the calculated value of 45,495 Da based on the 410 amino acids, which includes the 6 histidines of
3 the affinity tag. The molecular weight of CcBgl1A is similar to that of some GH1
4 ginsenoside-hydrolyzing β-glucosidases, such as *A. chlorophenolicus* (43.7 kDa) [23], *P.*
5 *mucilaginosus* (~48 kDa) [21], *Sphingomonas* sp. 2F2 (50 kDa) [14], *S. alaskensis* (51 kDa) [9],
6 and *P. furiosus* (55.5 kDa) [20].
7 With native PAGE and MUG zymogram analysis (Fig. 1b), the single band stained by
8 Coomassie Brilliant Blue G-250 was at the same location as the β-glucosidase activity band,
9 which proved the recombinant CcBgl1A is indeed a β-glucosidase.
10 *3.3 Characterization of Recombinant β-Glucosidase CcBgl1A from C. cellulans sp. 21*
11 The hydrolytic activity of CcBgl1A was examined over a pH range from 2.0 to 11.0 with
12 *p*NPβGlc as a substrate (Fig. 2). A maximum activity was observed at pH 5.5. CcBgl1A was stable
13 within the pH range from 6.0 to 10.0. After pre-incubated at 4°C for 24 h, over 60% of the activity
14 was retrieved at pH 6.0-10.0 (Fig. 2). The effect of temperature on the enzyme activity was
15 investigated at pH 5.5, and a maximum activity was recorded at 35°C (Fig. 3a). The effect of
16 temperature on enzyme stability was investigated by varying the temperature from 30 to 50°C at a
17 constant pH of 5.5. The results showed that CcBgl1A exhibited a poor thermal stability. More than
18 80% of its activity remained after treatment at temperature below 30°C for 1 h, however, the
19 activity significantly decreased above 35°C and was completely lost at 50°C for 5 min incubation
20 (Fig. 3b). The half-life values were 13.5 min, 7.6 min, 5.1 min and 3.4 min for 35, 40, 45 and
21 50°C. Therefore, for the efficiency of biotransformation, the reaction temperature was determined
22 to be 30°C.

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1 The effects of metal ions and reagents on CcBgl1A activity were also investigated (Table 2).
2 CcBgl1A activity was not affected by most metal ions and reagents we detected at the
3 concentration of 5 mM, only Hg^{2+} and SDS significantly inhibited the enzymatic activity of
4 CcBgl1A. In contrast, EDTA and Mg^{2+} slightly activated the enzymatic activity of CcBgl1A. At
5 the concentration of 50 mM, most metal ions and reagents inhibited the activity of CcBgl1A.
6 *3.4 Substrate Specificity of CcBgl1A on pNP Glycosides and Disaccharides*
7 The substrate specificity of β-glucosidase CcBgl1A from *C. cellulans* sp. 21 was investigated
8 using aryl-glycosides, disaccharides, and ginsenosides (Table 3). When using aryl-glycosides as
9 substrates, the hydrolysis order was *p*NPβGlc >> *p*NPβXyl > *p*NPαAra*f* ≈ *p*NPβMan > *p*NPβGal.
10 The *p*NP-α-glycosides we tested were not substrates for CcBgl1A. The GH1 enzyme CcBgl1A
11 had good activity on sophorose (glucose-β-(1→2)-glucose), medium activity on laminaribiose
12 (glucose-β-(1→3)-glucose), but very weak activity on cellobiose (glucose-β-(1→4)-glucose), and
13 no activity on gentiobiose (glucose-β-(1→6)-glucose). The linkage preference for disaccharides
14 was β-1, 2 > β-1, 3 >>> β-1, 4.
15 Based on substrate specificity, β-glucosidases are classified as aryl β-glucosidases, that act on
16 aryl-glucosides; true cellobiases, that catalyze the hydrolysis of cellobiose to release glucose; and
17 broad substrate specificity β-glucosidases, that act on a variety of substrates [25]. CcBgl1A
18 showed significant activity for both aryl-glucosides *p*NPβGlc and glucose disaccharides,
19 indicating that it belongs to broad substrate specificity β-glucosidase. Enzymes from GH family 1
20 are reported to be good candidates for true cellobiase in a variety of microorganisms [26-28].
21 However, our results showed that CcBgl1A is a good β-glucosidase, but not an efficient cellobiase,
22 as it showed much better activity on β-1, 2 linked glucose (sophorose) and β-1, 3 linked glucose

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1 (laminaribiose) than β-1, 4 (cellobiose).

2 *3.5 Substrate Specificity of β-Glucosidase CcBgl1A on PPD-type Ginsenosides*

3 When using a PPD-type ginsenosides mixture PPDGM as the substrate, the enzyme showed
4 considerable activity on protopanaxadiol-type ginsenosides Rb1, Rb2, Rc and Rd. TLC and HPLC
5 analysis revealed that in the presence of CcBgl1A, PPDGM including ginsenosides Rb1, Rb2, Rc
6 and Rd were converted into different metabolites, suggesting that CcBgl1A is a
7 ginsenoside-hydrolyzing enzyme (Fig. 4). To verify the bioconversion pathway and efficiency of
8 CcBgl1A, each individual ginsenoside was used as substrates for the biotransformation, and
9 HPLC analyses were carried out for products determination. As shown in Fig. 5, the products were
10 Gyp XVII for Rb1, compound O for Rb2, compound Mb for Rc, and F2 for Rd. The results
11 indicated that the enzyme only removed the outer glucose linked to the β-1,2 linkage of C-3
12 position in ginsenosides Rb1, Rb2, Rc, and Rd. The inner glucose linked to the C-3 position and
13 the disaccharides at the C-20 position with a β-1,6 linkage were not attacked by CcBgl1A (Fig. 6).
14 It is noteworthy that although CcBgl1A exhibited some activity on *p*NPαAra*f*, it showed no
15 hydrolysis activity on the α-Ara*f* (1→6) Glc- in the C-20 position of ginsenoside Rc, therefore, no
16 metabolite Rd or F2 was observed even by prolonging the reaction time of CcBgl1A with
17 ginsenoside Rc to 12 h (data not shown). The reason for this might be that the spatial
18 conformation of ginsenoside molecule blocked the attack of enzyme to the α-Ara*f* (1→6) Glc-
19 linkage.

20 In previous reports, some bacterial GH1 ginsenoside-hydrolyzing β-glucosidases were
21 heterologous expressed in *E. coli* (Table S1). The hydrolysis behavior of CcBgl1A on
22 ginsenosides was similar to the GH1 β-glucosidase from *S. alaskensis* [9], although they shared

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1 low amino acid sequence similarity (33%). However, CcBgl1A and the *S. alaskensis*
2 β-glucosidase possessed different physicochemical properties, especially the molecular weight,
3 optimal temperature, and substrate specificity on *p*NP compounds. GH1 enzyme BglPm from *P.*
4 *mucilaginosus* also hydrolysed ginsenoside Rb1 to Gyp XVII, and hydrolysed ginsenoside Rd to
5 F2. However, Gyp XVII was not the final metabolite, which would be further converted to F2 [21].
6 GH1 enzyme BglSp from *Sphingomonas* sp. 2F2 also converted Rb1, Rb2, and Rc to Gyp XVII,
7 compound O, and compound Mb, respectively, which was further converted to F2 by hydrolyzing
8 the outer glycoside linkage at the C-20 position [14]. Other GH family 1 β-glucosidases from *A.*
9 *chlorophenolicus*, *P. furiosus*, *S. solfataricus*, and *S. acidocaldarius* hydrolysed the sugar moieties
10 at both the C-3 and C-20 positions in PPD-type ginsenosides Rb1, Rb2, Rc [11, 19, 20, 23].

11 *3.6 Kinetic Parameters of CcBgl1A*

12 The kinetic parameters of CcBgl1A on different substrates were determined, as shown in Table
13 4. The catalytic efficiencies (k_{cat}/K_m) for the substrates decreased in the following order:
14 *p*NPβGlc > Rb1 > Rb2 > Rc > sophorose > Rd > laminaribiose > *p*NPβXyl. CcBgl1A had a
15 comparable K_m value for *p*NPβGlc (0.53 mM), lower than that of GH1 β-glucosidases from
16 bacteria, such as *P. mucilaginosus* (3.24 mM) [21], *Sphingomonas* sp. 2F2 (2.9 mM) [14],
17 *Sphingomonas paucimobilis* (1.3 mM) [29], and fungi including *Neosartorya fischeri* (2.8 mM)
18 [30], *Piromyces* sp. E2 (1 mM) [31] and *Stereum hirsutum* (2.5 mM) [32]. The catalytic
19 efficiencies for hydrolysis ginsenosides by CcBgl1A were higher than those of β-glycosidase from
20 *S. acidocaldarius* for ginsenosides Rd (4.8 $mM^{-1}min^{-1}$) and Rb1 (4.8 $mM^{-1}min^{-1}$) [19], and
21 β-glucosidase from *P. mucilaginosus* for Rb1 (1.23 $mM^{-1}s^{-1}$), Rd (1.78 $mM^{-1}s^{-1}$), and Gyp XVII
22 (1.17 $mM^{-1}s^{-1}$) [21].

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1 *3.7 Scaled-up Production of Minor Ginsenosides by Crude Recombinant CcBgl1A*

2 PPDGM was used as substrate for the mass production of minor ginsenosides Gyp XVII,
3 compound O, compound Mb and F2. After biotransformation, 1 g PPDGM yielding 292 ± 12 mg
4 Gyp XVII, 134 ± 5.8 mg compound O, 184 ± 7 mg compound Mb, and 62 ± 3.5 mg F2 with
5 chromatographic purities. The transformation was completely converted in 2 h, which is faster
6 than the microbial transformations (usually 24 h or even longer). To confirm the structure, each
7 product was identified by ^{13}C NMR. The ^{13}C NMR spectra of the obtained compounds (Table S2)
8 were consistent with Gyp XVII, compound O, compound Mb and F2 reported in the literatures.

9 Comparing to the other enzymes, this biotransformation would be potentially useful because of
10 following advantages: (i) The rare ginsenosides were produced using one recombinant enzyme,
11 which was easier to prepare than native enzymes from natural sources, and more stable than the
12 microbial biotransformation. (ii) The substrate PPDGM for biotransformation, which is relatively
13 abundant in total ginsenosides, did not need to be further separated for the minor ginsenosides
14 preparation. (iii) The recombinant CcBgl1A did not need to be purified, the crude recombinant
15 CcBgl1A was used for biotransformation. This high yields and simple procedure indicated that the
16 biotransformation has potential for industrial application. Further work are still needed for the
17 enlargement of the recombinant CcBgl1A transformation system to adapt it to the industrial
18 production.

19

20 **4. Conclusions**

21 In conclusion, we describe here the cloning of a ginsenoside-hydrolyzing β-glucosidase gene
22 belonging to glycoside hydrolase family 1 from *C. cellulans* sp.21. This enzyme was successfully

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overexpressed in *E. coli* and the recombinant enzyme was found to be specific to β-1, 2-glucosidic linkage in protopanaxadiol type ginsenosides, which transformed ginsenoside Rb1, Rb2, Rc and Rd to minor ginsenosides Gyp XVII, compound O, compound Mb and F2. Amounts of minor glycosides in the range of 62-292 mg were produced with the simple recombinant enzyme biotransformation.

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References

[1] D. Kiefer, T. Pantuso, Am. Fam. Physician 68 (2003) 1539-1542.

[2] T.K. Lee, R.M. Johnke, R.R. Allison, K.F. O'Brien, LJ.Jr. Dobbs, Mutagenesis 20 (2005) 237-243.

[3] C.L. Saw, A.Y. Yang, D.C. Cheng, S.S. Boyanapalli, Z.Y. Su, T.O. Khor, S. Gao, J. Wang, Z.H. Jiang, A.N. Kong, Chem. Res. Toxicol. 25 (2012) 1574-1580.

[4] J.D. Park, D.K. Rhee, Y.H. Lee, Phytochem. Rev. 4 (2005) 159-175.

[5] K.W. Leung, A.S. Wong, Chin. Med. 5 (2010) 20-26.

[6] C.S. Yuan, J.A. Wu, J. Osinski, Am. J. Clin. Nutr. 75 (2002) 600-601.

[7] L.P. Christensen, Adv. Food Nutr. Res. 55 (2009) 1-99.

[8] Q.F. Xu, X.L. Fang, D.F. Chen, J. Ethnopharmacol. 84 (2003) 187-192.

[9] K.C. Shin, D.K. Oh, J. Biotechnol. 172 (2014) 30-37.

ACCEPTED MANUSCRIPT

1 [10] E.A. Bae, M.J. Han, E.J. Kim, D.H. Kim, Arch. Pharm. Res. 27 (2004) 61-67.

2 [11] K.H. Noh, J.W. Son, H.J. Kim, D.K. Oh, Biosci. Biotechnol. Biochem. 73 (2009) 316-321.

3 [12] J. Gao, W.J. Xu, F. Liang, M. Hao, X.Y. Liu, L. Fu, R.T. Jin, N. Wang, G.H. Tai, Y.F. Zhou,

4 Ann. Microbiol. 63 (2013) 139-149.

5 [13] D.S. An, C.H. Cui, H.G. Lee, L. Wang, S.C. Kim, S.T. Lee, F. Jin, H. Yu, Y.W. Chin, H.K. Lee,

6 W.T. Im, S.G. Kim, Appl. Environ. Microbiol. 76 (2010) 5827-5836.

7 [14] L. Wang, Q.M. Liu, B.H. Sung, D.S. An, H.G. Lee, S.G. Kim, S.C. Kim, S.T. Lee, W.T. Im, J.

8 Biotechnol. 156 (2011) 125-133.

9 [15] J.K. Kim, C.H. Cui, M.H. Yoon, S.C. Kim, W.T. Im, J. Biotechnol. 161 (2012) 294-301.

10 [16] C.H. Cui, S.C. Kim, W.T. Im, Appl. Microbiol. Biotechnol. 97 (2013) 649-659.

11 [17] C.S. Park, M.H. Yoo, K.H. Noh, D.K. Oh, Appl. Microbiol. Biotechnol. 87 (2010) 9-19.

12 [18] X.D. Yang, Y.Y. Yang, D.S. Ouyang, G.P. Yang, Fitoterapia 100C (2015) 208-220.

13 [19] K.H. Noh, D.K. Oh, Biol. Pharm. Bull. 32 (2009) 1830-1835.

14 [20] M.H. Yoo, S.J. Yeom, C.S. Park, K.W. Lee, D.K. Oh, Appl. Microbiol. Biotechnol. 89 (2011)

15 1019-1028.

16 [21] C.H. Cui, J.K. Kim, S.C. Kim, W.T. Im, PLoS One 9 (2014) e85727.

17 [22] J. Du, C.H. Cui, S.C. Park, J.K. Kim, H.S. Yu, F.X. Jin, C. Sun, S.C. Kim, W.T. Im, PLoS

18 One 9 (2014) e96914.

19 [23] M.K. Park, C.H. Cui, S.C. Park, S.K. Park, J.K. Kim, M.S. Jung, S.C. Jung, S.C. Kim, W.T.

20 Im, J. Microbiol. 52 (2014) 399-406.

21 [24] G.L. Miller, Anal. Chem. 31 (1959) 426-428.

22 [25] Y. Bhatia, S. Mishra, V.S. Bisaria, Crit. Rev. Biotechnol. 22 (2002) 375-407.

Accepted Manuscript

ACCEPTED MANUSCRIPT

1 [26] D.R. Love, R. Fisher, P.L. Bergquist, Mol. Genet. Genomics 213 (1988) 84-92.

2 [27] F. Grabnitz, M. Seiss, K.P. Rucknagel, W.L. Staudenbauer, Eur. J. Biochem. 200 (1991)

3 301-309.

4 [28] J. Gao, W. Wakarchuk, J. Bacteriol. 196 (2014) 4103-4110.

5 [29] A.R. Marques, P.M. Coutinho, P. Videira, A.M. Fialho, I. Sá-Correia, Biochem. J. 370 (2003)

6 793-804.

7 [30] P. Ramachandran, M.K. Tiwari, R.K. Singh, J.R. Haw, M. Jeya, J.K. Lee, Process Biochem.

8 47 (2012) 99-105.

9 [31] H.R. Harhangi, P.J. Steenbakkers, A. Akhmanova, MS.M. Jetten, C. van der Drift, H.J. Op

10 den Camp, Biochim. Biophys. Acta 1574 (2002) 293-303.

11 [32] N.P. Nguyen, K.M. Lee, K.M. Lee, I.W. Kim, Y.S. Kim, M. Jeya, J.K. Lee, Appl. Microbiol.

12 Biotechnol. 87 (2010) 2107-2116.

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1 **Figure captions**

2 **Fig. 1** Electrophoresis analysis of CcBgl1A. (a) SDS-PAGE analysis on 10% resolving gel. Lane 1,
3 culture lysate of *E. coli* BL21-pET28a-ccbgl1a before IPTG induction; lane 2, culture lysate of *E.*
4 *coli* BL21-pET28a-ccbgl1a after IPTG induction; lane 3, CcBgl1A purified from Ni sepharose
5 fastflow column; M, molecular weight marker (PageRuler Prestained Protein Ladder, Thermo
6 Scientific); (b) native PAGE of CcBgl1A with Coomassie blue G-250 staining (1) and
7 β-glucosidase activity staining with MUG (2).

8 **Fig. 2** Effect of pH on activity (solid symbols) and stability (hollow symbols) of CcBgl1A. The
9 optimal pH of CcBgl1A was determined by ranging pH from 2.0 to 11.0 using following buffers:
10 sodium acetate buffer, pH 2.0-6.0; Na_2HPO_4-NaH_2PO_4 buffer, pH 6.0-8.0; Glycine-NaOH buffer,
11 pH 8.0-11.0. The maximum activity obtained was defined as 100%. The pH stability of CcBgl1A
12 was determined by pre-incubating CcBgl1A in different pH for 24 h at 4°C and then determining
13 the percentage of residual activity under standard assay conditions. The activity of CcBgl1A
14 without pre-incubating was defined as 100%. Results are presented as means ± standard deviations
15 (n = 3).

16 **Fig. 3** Effect of temperature on activity (a) and stability (b) of CcBgl1A using *p*NPβGlc as
17 substrate. The optimal temperature (a) was determined at different temperatures from 20 - 80°C.
18 The maximum activity obtained was defined as 100% activity. Thermal stability was determined
19 by incubating the enzyme for 1 h at different temperatures. The activity of the enzyme before
20 incubation was defined as 100%. Results are presented as means ± standard deviations (n = 3).

21 **Fig. 4** Analysis of ginsenosidase activity of CcBgl1A on PPDGM by TLC (a) and HPLC (b). (a): 1,
22 biotransformation substrate PPDGM; 2, transformed products of PPDGM; S, standards; (b): 1,

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1 biotransformation substrate PPDGM; 2, transformed products of PPDGM; 3, standards.

2 **Fig. 5** HPLC analysis of the hydrolysis products of single ginsenosides by CcBgl1A. Purified
3 CcBgl1A was incubated with single Rb1, Rb2, Rc and Rd, respectively. The reaction was
4 terminated by adding equal volume of n-butanol. The n-butanol fraction was subjected to HPLC
5 analysis as described in Methods section. Peak 1-8 represents ginsenosides Rb1, Rb2, Rc, Rd, Gyp
6 XVII, compound O, compound Mb and F2, respectively, the retention times of which were
7 determined to be 15.2 min, 22.1 min, 18.4 min, 26.1 min, 28.1 min, 30.9 min, 30.2 min and 35.2
8 min, respectively.

9 **Fig. 6** Biotransformation pathways of ginsenosides Rb1, Rb2, Rc and Rd by recombinant
10 CcBgl1A.

11

12 • A new GH1 gene *ccbgl1a* from *C. cellulans* sp. 21 was cloned and expressed in *E. coli*.

13 • Enzyme CcBgl1A was specific to β-1,2- glycosidic bond at C-3 position of ginsenosides.

14 • CcBgl1A was applied for one-pot preparation of ginsenoside GXVII, CO, Mb and F2.

15

16 **Table 1** Summary of purification of recombinant CcBgl1A

Purification step	Volume (l)	Total Protein (g)[a]	Total activity (kU)[b]	Sp act (U/mg)	Recovery (%)	Purification (fold)
Crude enzyme extract	1.2	0.60	10.0	16.7	100	1.0
Ni sepharose fastflow column	0.02	0.23	7.2	31.5	72	1.9

17 [a]Protein was quantified according to the Bradford method using bovine serum albumin (BSA) as standard.

18 [b]The activity was reported as activity on *p*NPβGlc.

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2 **Table 2** Effects of metal ions and chemical agents on the activity of CcBgl1A

Metal ions or reagents	Relative activity (%)[a]	
	5 mM	50 mM
K^{+}	105.5 ± 5.2	79.4 ± 2.3
Mg^{2+}	110.6 ± 2.8	38.2 ± 1.5
Ca^{2+}	108.4 ± 2.4	11.3 ± 0.5
Na^{+}	109.0 ± 2.9	96.0 ± 0.5
Cu^{2+}	91.1 ± 1.5	2.1 ± 0.7
Fe^{3+}	91.3 ± 2.1	_[b]
Hg^{2+}	15.5 ± 8.4	10.8 ± 0.8
Ba^{2+}	106.7 ± 1.4	7.8 ± 0.3
Mn^{2+}	87.5 ± 2.9	10.4 ± 0.2
DTT	104.6 ± 2.1	91.9 ± 8.6
EDTA	121.2 ± 1.6	7.7 ± 0.4
SDS	_	_

3 [a]The activity assayed in the absence of cations or reagents was taken as 100%. Results are presented as means ±

4 standard deviations (n = 3).

5 [b]-: Not detected.

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2 **Table 3** Relative activity of CcBgl1A on different substrates[a]

Substrate[b]	Relative activity (%)[c]	Substrate	Relative activity (%)
*p*NPβGlc	100 ± 0.0	sophorose	100 ± 0.0
*p*NPβXyl	30.8 ± 0.6	laminaribiose	51.5 ± 0.9
*p*NPβMan	7.2 ± 0.1	cellobiose	5.9 ± 0.1
*p*NPβGal	1.4 ± 0.1	gentiobiose	0
*p*NPαAra*f*	9.5 ± 0.5	Rb1	100 ± 0.0
*p*NPαGlc	0.7 ± 0.2	Rb2	100 ± 0.0
*p*NPαGal	0.6 ± 0.1	Rc	87.5 ± 0.1
*p*NPαMan	0.4 ± 0.1	Rd	66.8 ± 0.2

3 [a] Reactions were performed with 5 mM substrate, pH 6.0, at 30°C for 10 min.

4 [b]Absorption caused by released *p*-nitrophenol or reducing sugars was measured at 405 or 520 nm. The hydrolysis

5 product of ginsenosides was measured by HPLC. The relative activity on *p*NPβGlc (31.5 μmol/min/mg), sophorose

6 (19.3 μmol/min/mg) and ginsenoside Rb1 (7.5 μmol/min/mg) was taken as 100%, respectively.

7 [c] The data are reported as means ± standard errors from the mean for three independent experiments.

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2 **Table 4** Kinetic parameters of CcBgl1A on different compounds

Substrate[a]	K_m (mM)[b]	Vmax (μmol/min/mg)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
*p*NPβGlc	0.53 ± 0.04	39.71 ± 0.37	30.08 ± 0.28	57.16 ± 3.37
*p*NPβXyl	1.34 ± 0.03	0.34 ± 0.05	0.26 ± 0.04	0.19 ± 0.02
Sophorose	1.89 ± 0.15	26.57 ± 0.84	20.37 ± 0.64	10.84 ± 0.49
Laminaribiose	4.89 ± 0.69	38.12 ± 2.72	29.23 ± 2.09	6.04 ± 0.43
Ginsenoside Rb1	0.26 ± 0.03	11.10 ± 0.42	8.52 ± 0.33	32.77 ± 1.29
Ginsenoside Rb2	0.15 ± 0.01	6.10 ± 0.05	4.67 ± 0.04	31.44 ± 1.63
Ginsenoside Rc	0.22 ± 0.01	6.93 ± 0.13	5.31 ± 0.10	24.16 ± 0.64
Ginsenoside Rd	1.24 ± 0.03	13.91 ± 0.11	10.64 ± 0.08	8.64 ± 0.11

3 [a]The hydrolysis rates for CcBgl1A with *p*NPβMan and *p*NPβGal were too low to calculate the kinetics parameters,

4 even when increasing the enzyme concentration to 1 mg/ml in the reaction mixture.

5 [b]The data are reported as means ± standard errors from the mean for three independent experiments.

6

7

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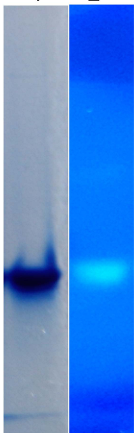
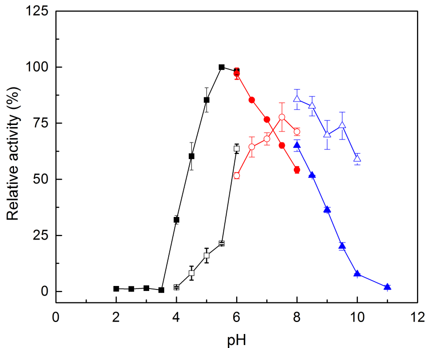
a

kDa M 1 2 3

170
130
100
70
55
40
35
25





125
100
75
50
25
0
Relative activity (%)
0
2
4
6
8
10
12
pH

a

Relative activity (%)

Tempreture(°C)

b

Relative activity (%)

Time (min)

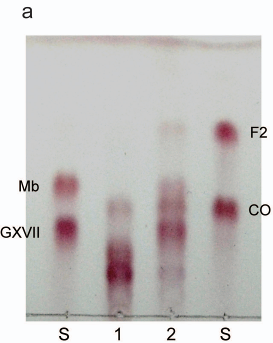
30℃

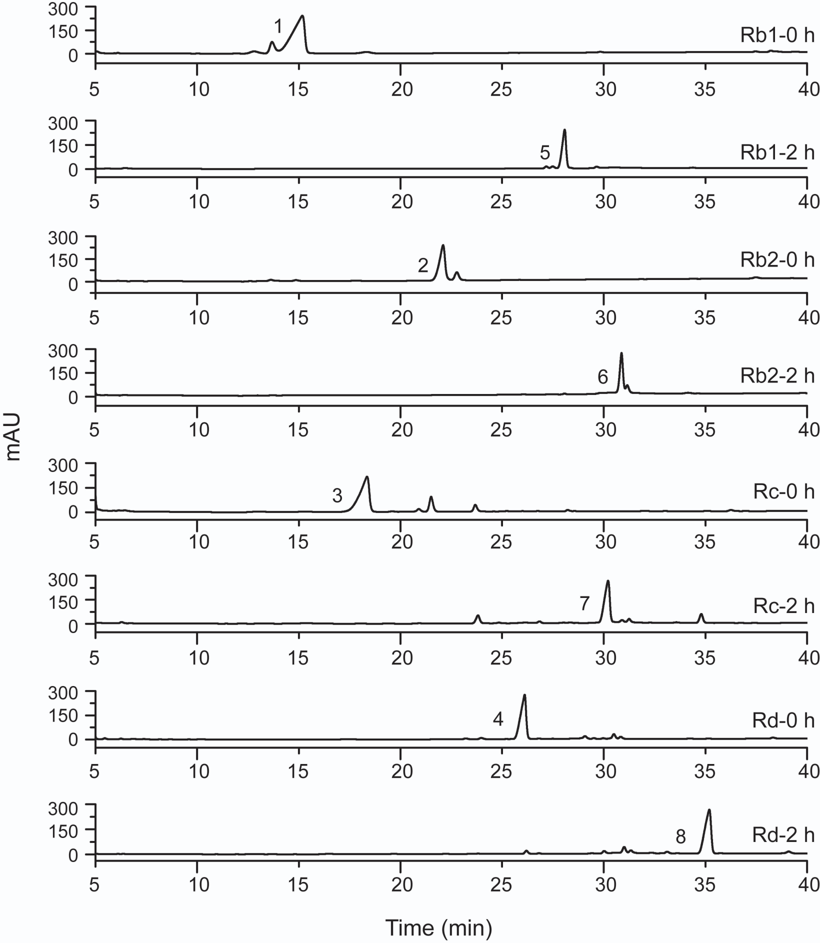
35℃

40℃

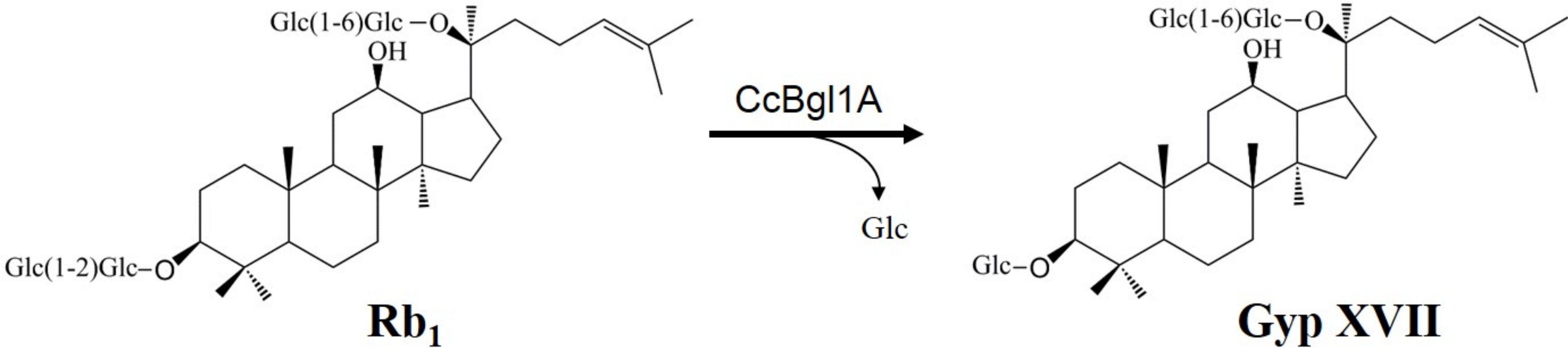
45℃

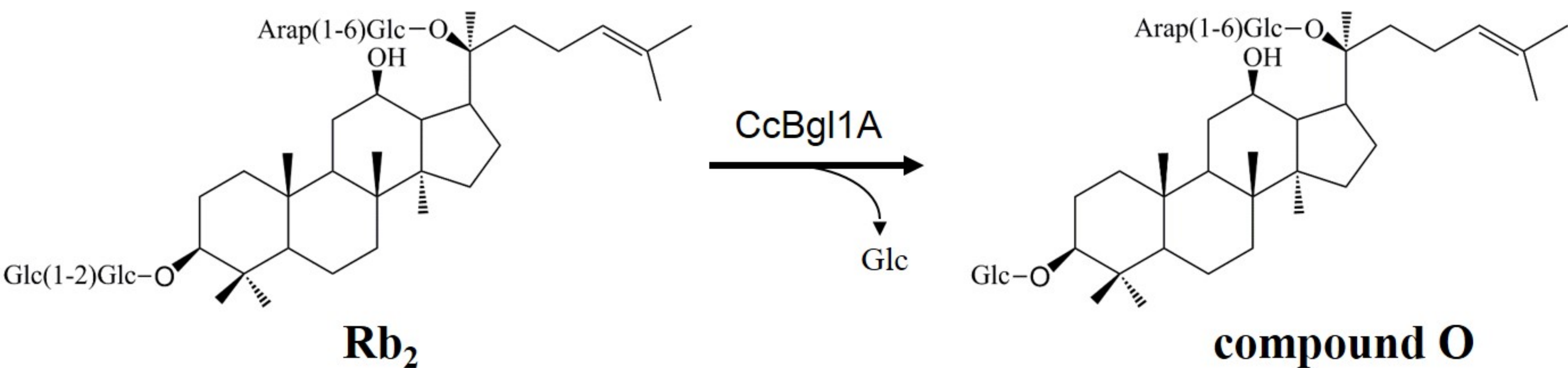
50 ℃


a
Mb
GXVII
F2
CO
S
1
2
S
b
300
150
0
0
10
20
30
40
1
2
3
mAU
Rb1
Rc
Rb2
Rd
GXVII
Mb
CO
F2
Time (min)



Rb1-0 h
Rb1-2 h
Rb2-0 h
Rb2-2 h
Rc-0 h
Rc-2 h
Rd-0 h
Rd-2 h
1
2
3
4
5
6
7
8
300
150
0
5
10
15
20
25
30
35
40
mAU
Time (min)


Glc(1-6)Glc−O
OH
Glc(1-2)Glc−O
Rb1
CcBgl1A
Glc
Glc(1-6)Glc−O
OH
Glc−O
Gyp XVII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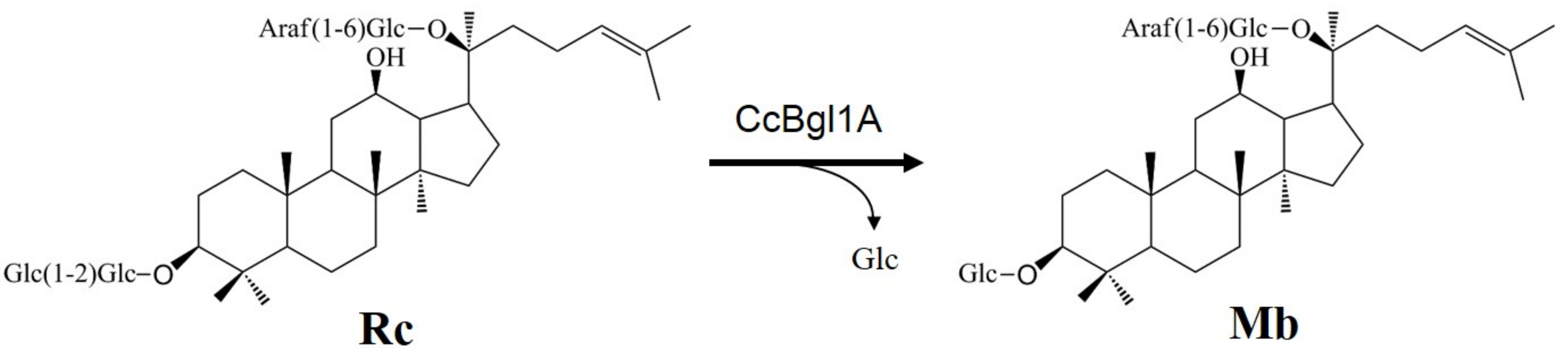
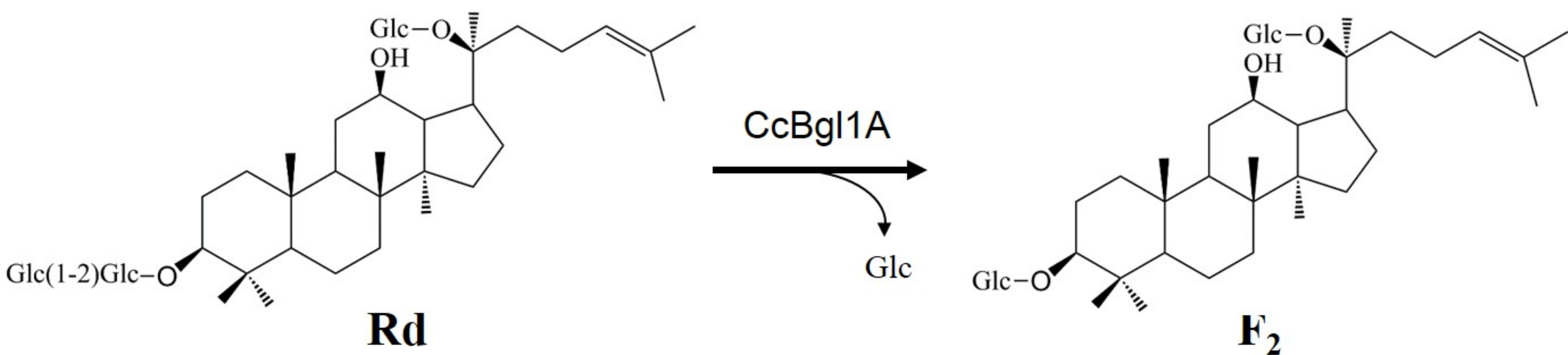
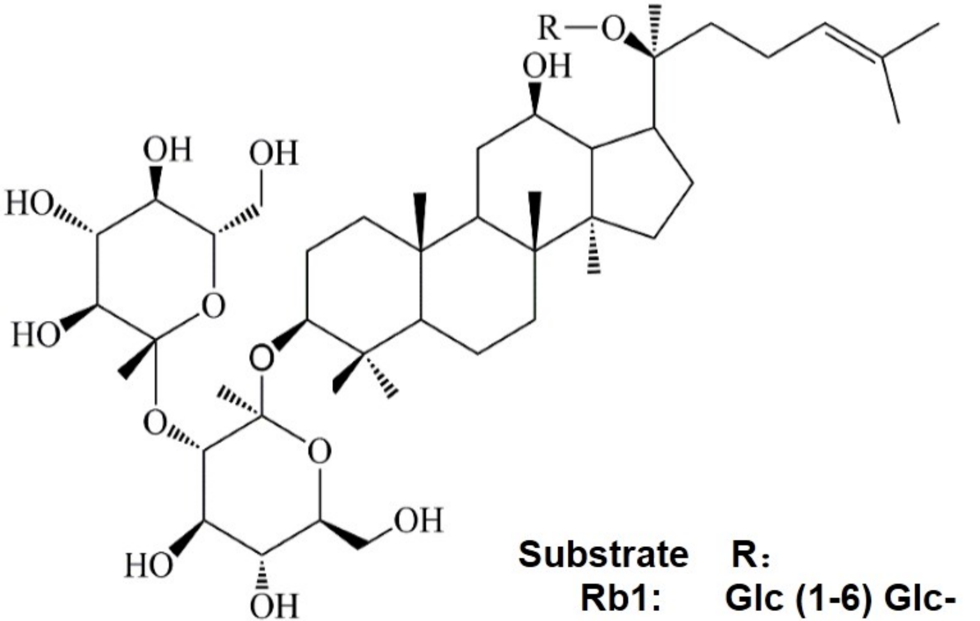
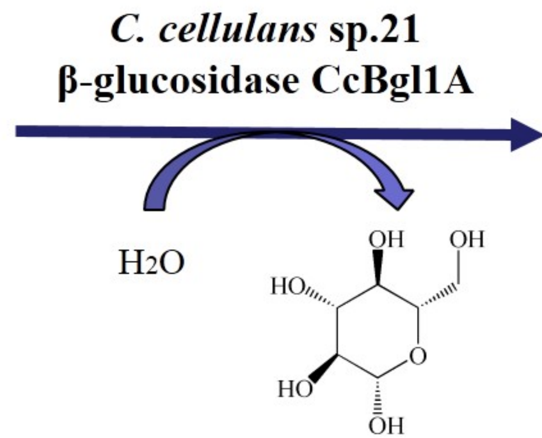
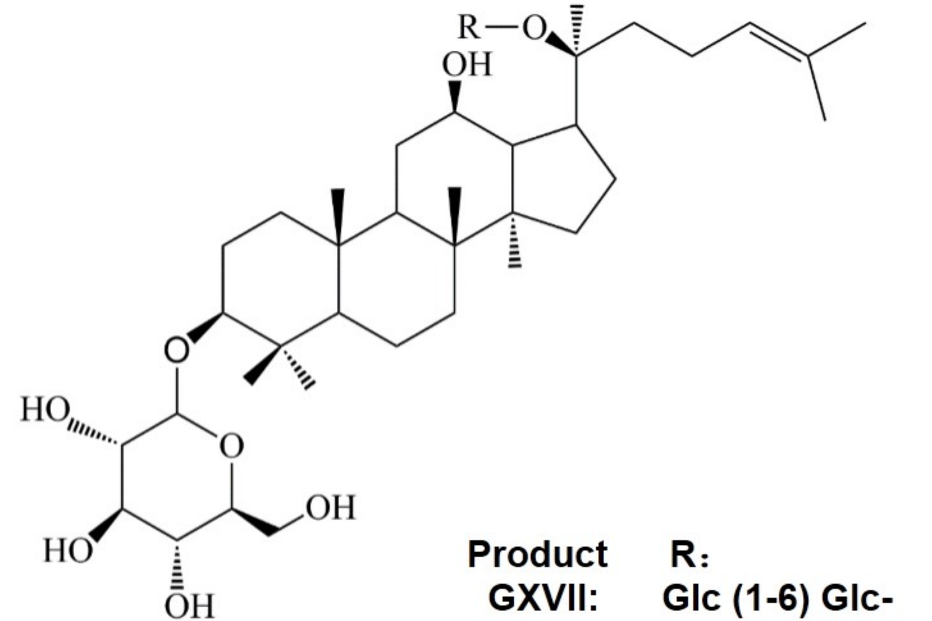
OH
Glc(1-2)Glc−O
Rb2
CcBgl1A
Glc
Arap(1-6)Glc−O
OH
Glc−O
compound O


Araf(1-6)Glc−O
OH
Glc(1-2)Glc−O
Rc
CcBgl1A
Glc
Araf(1-6)Glc−O
OH
Glc−O
Mb


Glc−O
OH
Glc(1-2)Glc−O
Rd
CcBgl1A
Glc
Glc−O
OH
Glc−O
F2


R—O
OH
OH
OH
HO
O
HO
O
O
O
OH
HO
OH
Substrate R:
Rb1: Glc (1-6) Glc-
Rb2: Arap (1-6) Glc-
Rc: Araf (1-6) Glc-
Rd: Glc-


C. cellulans sp.21
β-glucosidase CcBgl1A
H2O
OH
OH
HO
O
HO
OH


R—O
OH
O
HO
O
OH
HO
OH
Product R:
GXVII: Glc (1-6) Glc-
CO: Arap (1-6) Glc-
Mb: Araf (1-6) Glc-
F2: Glc-