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ORIGINAL ARTICLE

A novel ginsenoside-hydrolyzing enzyme from *Penicillium oxalicum* and its application in ginsenoside Rd production

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Abstract

The fungus *Penicillum oxalicum* can selectively metabolize the major 20(S)-protopanaxadiol ginsenosides Rb1, Rb2, and Rc using extracellular glycosidases yielding a series of bioactive metabolites. A β-glucosidase GH1 was purified from the culture of *P. oxalicum* with a yield of 9.5% and a specific activity of 3.9×10^3 U/mg. GH1 was a tetramer with a native molecular weight of 484 kDa and its *p*I value was pH 4.2. GH1 specifically cleaved the β-(1-6)-glucosidic linkage at C-20 site of ginsenoside Rb1 to give the sole product Rd. The optimum conditions were established to be pH 4.5, 55°C, and 0.25 U/ml purified enzyme at 2 mg/ml ginsenoside Rb1. GH1 could be used in the pharmaceutical industry.

Keywords: *Ginsenoside Rb1, ginsenoside Rd,* β*-glucosidase, Penicillium oxalicum, biotransformation*

Introduction

Ginseng has been used as a medicine in the world for its various medical applications and biological or pharmacological properties including immunomodulation, adaptogenic, anti-fatigue, and anti-aging effects (Zhang et al. 1994; Borchers et al. 1998; Kiefer & Pantuso 2003; Lee et al. 2005; Saw et al. 2012). Triterpene glycosides found in ginseng, known as ginseng saponins or ginsenosides, are reported to be the compounds responsible for the biological and pharmacological activities of ginseng (Attele et al. 1999; Yuan et al. 2002). Ginsenoside Rd has various bioactivities such as the protection of neurons from neurotoxins, the enhancement of neural stem cell differentiation, and the prevention of blood vessel contraction (Lee et al. 2003; Zeng et al. 2003; Shi et al. 2005). The amount of Rd in ginseng roots is about 4–11%, which is much less than Rb1. Rd can be produced by hydrolysis of the 20-C-outer glucosidic residue of Rb1 (Figure 1). The hydrolysis of Rb1 can be performed by acidic or enzymatic catalysis. Enzymatic catalysis is considered a more promising method due to its high selectivity, mild reaction conditions, and environmental compatibility. Although the production of pharmaceutically active ginsenoside Rd has been performed from ginsenoside Rb1 or Rc by several microorganisms or enzymes (Zhang et al. 2002; Hu et al. 2007; Ko et al. 2007; Zhao et al. 2009), we still need to explore new sources and new enzymes with high specificity and yield in ginsenoside Rd production.

In our previous work, the fungus *P. oxalicum* was shown to transform protopanaxadiol-type ginsenosides (Gao et al. 2013). An enzyme mixture was prepared from the culture filtrate of *P. oxalicum* and ginsenosides Rb1, Rb2, and Rc were transformed to the final product compound K *via* different pathways by the enzyme mixture. In this study, a β-glucosidase GH1 was further purified from the crude enzyme solution of *P. oxalicum* and its capacity to transform ginsenoside Rb1 was investigated.

Methods

Strain and ginsenoside substrates

The fungus *Penicillium oxalicum* was isolated and identified in our laboratory. Ginsenoside standards Rb1, Rc, Rb2, and Rd were prepared in our laboratory and identified by ^{13}C-NMR. All other materials

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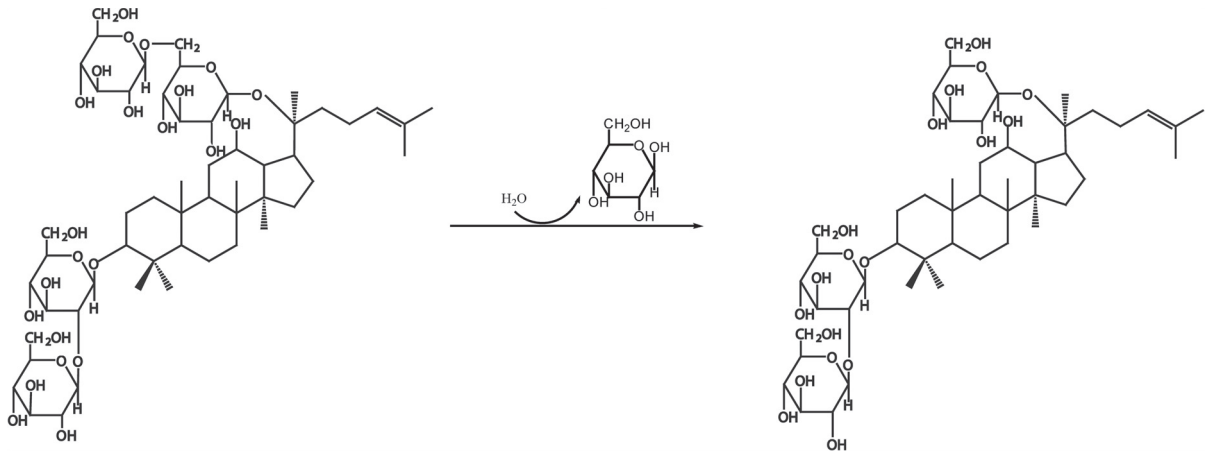

Figure 1. Production of ginsenoside Rd from ginsenoside Rb1.

and reagents were from Sigma Aldrich (St. Louis, MO, US) and Pharmacia, Sweden.

Enzyme purification

P. oxalicum was isolated from ginseng-cultivation soil and identified by ITS sequencing. The enzyme mixture of *P. oxalicum* was prepared according to our previous work (Gao et al. 2013), applied to a Phenyl sepharose CL-4B column (1.5 × 10.2 cm), and then submitted to stepwise elution with 36 ml of 1.50, 1.25, 1.00, 0.75, 0.50, 0.25, and 0 M $(NH_4)_2SO_4$ in 20 mM NaAc buffer (pH 5.0). Three β-glucosidase activity peaks were observed in the elution profile, referred to as GH1, GH2, and GH3 (Figure 2a). GH1 was further purified on a Sepharose CL-6B (3.0 × 90 cm) column. The fractions of 270–340 ml with high activity were collected, dialyzed against 20 mM Tris-HCl, pH 6.8 (Figure 2b), and finally purified on a Q Sepharose fast flow (1.5 × 2 cm) column. The column was eluted with 7.5 ml of 0, 0.1, 0.2, 0.3, and 0.4 M NaCl in 20 mM Tris-HCl, pH 6.8 (Figure 2c). The fractions with enzyme activity were recovered for determination of purity and properties.

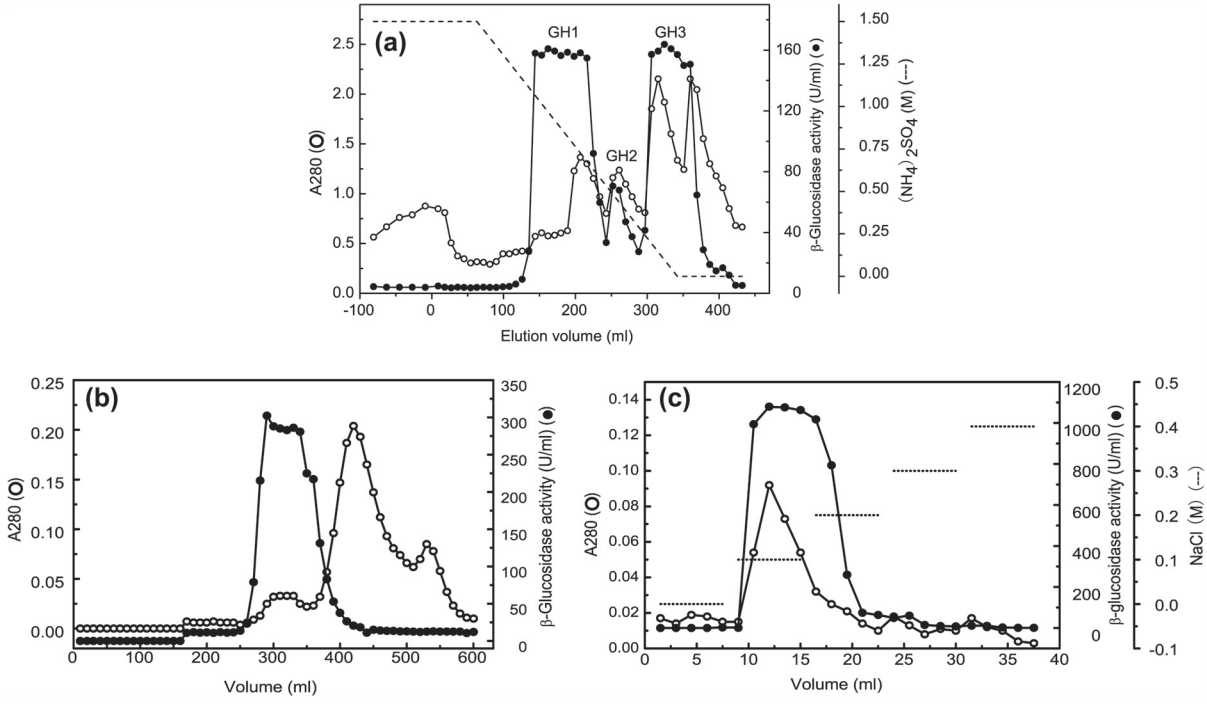

Figure 2. Elution profiles of GH1: (a) Phenyl Sepharose CL-4B column; (b) Sepharose CL-6B; (c) Q Sepharose fastflow; (●) represents β-glucosidase activity; (○ or —) represents absorption at 280 nm; (—) represents salt gradient.

Enzyme activity assay

β-Glucosidase activity was assayed using 10 mM *p*-nitrophenyl-glycosides (pNPGs) at pH 5.0 and 37°C (Zhao et al. 2009b). All other *p*NPGs were assayed under the same conditions. Enzyme activities against methyl-glycosides and disaccharides were determined using the dinitrosalicylic acid at 520 nm for the determination of reducing sugars (Miller 1959). Ginsenoside hydrolysis by GH1 was carried out with 1 mg of each ginsenoside in 0.5 ml buffer A. Aliquots (100 µl) were withdrawn at different time points and assayed by analytical HPLC system using a Shim-pack PREP-ODS (H) column (Gao et al. 2013). The content of product was calculated based on the peak area. One unit (U) of β-glucosidase activity was defined as the amount of enzyme liberating 1 nmol/min of *p*-nitrophenyl/reducing sugar/ginsenoside product under assay conditions.

Enzyme characterization

The purified enzyme was analyzed by SDS-PAGE on an 8% separating gel (Laemmli 1970) and stained with Coomassie Brilliant Blue G-250. After SDS-PAGE, the gel was stained with GelCode Glycoprotein Staining Kit (Pierce) to check if GH1 is a glycoprotein. The native molecular weight of GH1 was determined by gel filtration chromatography on Superose 6 10/300 GL column pre-calibrated with a molecular weight marker kit (MW-GF-1000 from Sigma) (Gao et al. 2010). Zymograms were carried out on 8% native PAGE, and enzyme bands visualized by incubating the gel in 20 mM acetate buffer containing 2 mM MUG at 25°C (Gao et al. 2010). The isoelectric point (*p*I) of GH1 was determined by the Model 111 Mini IEF Cell (Bio-Rad) and stained with Coomassie Brilliant Blue G-250.

The optimum pH of the purified enzyme was determined using 25 mM Na_2HPO_4-citrate buffer (pH 2.0–8.0), with *p*NPG as substrate. The pH stability of GH1 was determined using the following buffers: 25 mM Na_2HPO_4-citrate (pH 2.0–8.0), 25 mM glycine-NaOH (pH 8.0–11.0), and 25 mM Na_2HPO_4–NaOH buffer (pH 11.0–12.0). The temperature effect was analyzed between 20°C and 90°C at the optimum pH and the temperature stability was established from 20°C to 90°C.

Metal ions (various cations in the form of chlorides, final concentration of 50 mM), EDTA, or SDS (500 mM) were incubated with purified enzyme at 25°C and optimal pH for 1 h. After incubation, the residual activity was determined using *p*NPG as substrate. The activity of enzyme assayed in the absence of the chemical agents was set as 100%.

To determine the K*m* and V*max* values, the activity of GH1 was measured at its optimum pH and temperature with different concentrations of *p*NPG varying from 0.02 to 5 mM. The K*m* and V*max* values were calculated using a Lineweaver–Burk plot (Lineweaver & Burk 1934).

Production of ginsenoside Rd from ginsenoside Rb1 by GH1

To improve ginsenoside Rd productivity, biotransformation of Rb1 by GH1 was optimized. In a 50 ml reaction system, 100 mg ginsenoside Rb1 was incubated with purified GH1 at different pH, temperatures, and enzyme concentrations. Aliquots were withdrawn at different time points and assayed by separation on a C18 column as described in Methods. The transformation product was further identified by ^{13}C-NMR.

Results

Purification of GH1

The enzyme mixture was prepared by DEAE-cellulose chromatography and $(NH_4)_2SO_4$ precipitation from the culture filtrate of *P. oxalicum* (Gao et al. 2013). The mixture was separated on a Phenyl Sepharose CL-4B column, eluted with gradual concentrations of $(NH_4)_2SO_4$, to give three glucosidase activity peaks, referred as GH1, GH2, and GH3, respectively (Figure 2a).

GH1 was further purified on Sepharose CL-6B (Figure 2b) and then a Q Sepharose fast flow column (Figure 2c). GH1 showed a single peak on a Superose 6 column (Figure 3a) and a single protein band on SDS-PAGE (Figure 3b). GH1 was enriched approximately 328-fold and the yield was 9.5% (Table I). The specific activity was 3907.8 U/mg protein.

Characterization of GH1

The molecular weight of GH1 was estimated to be 484 kDa by gel filtration (Figure 3a) and 129 kDa by SDS-PAGE (Figure 3b), suggesting that GH1 is a tetramer, composed of four identical subunits. After SDS-PAGE treatment, the purified enzyme was stained with GelCode Glycoprotein Staining Kit to determine if it was a glycoprotein. As shown in Figure 3c, stained protein showed a band at the same location as that of GH1, indicating that GH1 was a glycoprotein. IEF-PAGE analysis demonstrated that GH1 was an acidic protein with a *p*I value of pH 4.2 (Figure 3f), which is typical of some extracellular β-glycosidases isolated from fungi,

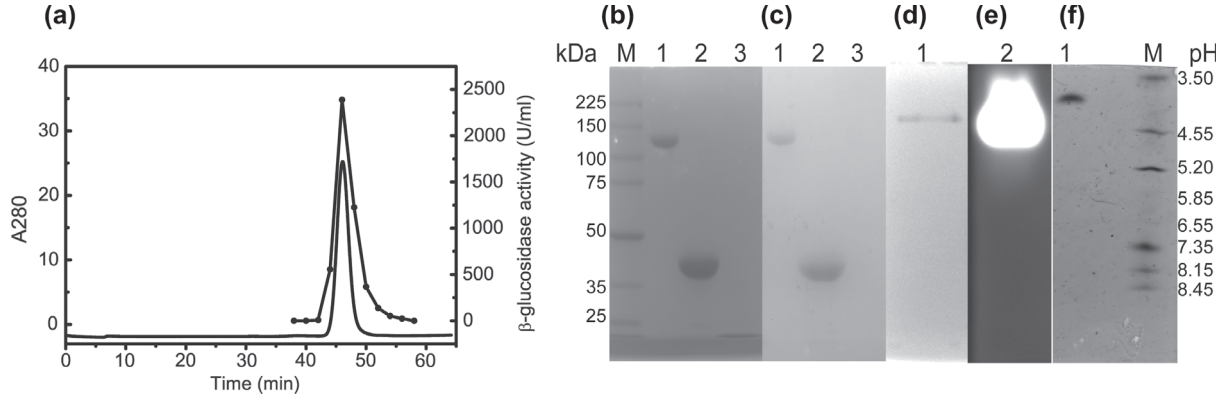

Figure 3. Analysis of GH1 by gel filtration (a) and Electrophoresis (b–f): SDS-PAGE with Coomassie blue staining (b) or PAS staining (c): M, protein marker; 1, GH1; 2, positive control; 3, negative control. Native PAGE with Coomassie blue staining (d) or MUG staining (e): 1, GH1. (f) IEF with Coomassie blue staining: M, marker; 1, GH1.

such as β-glycosidases from *Clavibacter michiganense* (*p*I 4.6) and *Trichoderma reesei* (*p*I 4.8) (Chen et al. 1992; Nakano et al. 1998). On native PAGE, the Coomassie brilliant blue-stained band for GH1 (Figure 3d) was at the same location as that using 4-MUG as substrate (Figure 3e), indicating that the band corresponded to β-glucosidase.

The temperature and pH dependence of purified GH1 are shown in Figure 4. The optimum pH for maximal GH1 activity was pH 3.5 (Figure 4a). Under optimal pH condition, the purified enzyme had a temperature optimum of 55°C (Figure 4c). The thermostability of the enzyme was investigated by measuring the residual activity after incubation at different times at temperatures ranging from 20 to 80°C. Under the conditions used, GH1 was highly stable at temperatures up to 60°C but was mostly inactivated at temperatures above 60°C (Figure 4d). The pH stability was also investigated, the enzyme was highly stable at pH 4.0–11.0, and 60% of the activity remained after incubation at pH 12.0 (Figure 4b).

The effects of chemical modifying agents and metal ions on GH1 are shown in Table II. None of the metal ions tested influenced the activity of GH1 significantly. In the presence of SDS at 0.5 M, the activity of GH1 was partially inhibited, with a relative activity of 40.88%.

Kinetic parameters of the purified enzymes were determined from Lineweaver–Burk plots with *p*NPG as substrate. The K*m* and V*max* values of GH1 were estimated to be 0.967 mM and 0.168 μmol/min/mg, respectively.

Table I. Purification of GH1 from *P. oxalicum*.

Procedure	Total activity (U)	Protein (mg)[a]	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme mixture	50033	3912.5	12.8	67.5	1
Phenyl Sepharose CL-4B	20198	1003.8	20.1	27.2	2
Sepharose CL-6B	10844	17.5	619.7	14.6	52
Q Sepharose fast flow	7034	1.8	3907.8	9.5	328

[a]Protein was quantified according to the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as standard.

Substrate specificity of GH1

The specificities of GH1 on *p*-nitrophenyl glycosides, methyl glycosides, disaccharides, and ginsenosides are summarized in Table III. While using aryl-glycosides as substrates, GH1 exhibited high specificity for β-D-glycoside compared with other *p*-nitrophenyl glycosides and methyl glycosides. If the activity against *p*NPG was defined as 100%, the activities were less than 5% against other *p*NPGs and methyl-α-glycosides. While using disaccharides as substrates, GH1 nearly hydrolyzed all the disaccharides tested with different activities, as shown in Table III. The hydrolysis order of sugars reflected the selectivity of GH1 on different glycosidic bonds. The hydrolysis preference toward the glycosidic bonds in the disaccharides was as follows: β-1, 2-glucosidic linkage > β-1, 6-glucosidic linkage > β-1, 4-glucosidic linkage > α-1, 1-glucosidic linkage > α-1, 2-glucosidic linkage > α-1, 4-glucosidic linkage. Besides, GH1 showed considerable activity against ginsenoside Rb1, no activity against ginsenosides Rb2, Rc, and Rd was demonstrated. The relative activity against Rb1 was 45.82% compared with that against *p*NPG.

Although GH1 exhibited high activity on the β-(1→2)-linked disaccharide sophorose, it showed no activity on β-(1→2)-glycosidic linkage in C-3 site of ginsenoside Rb1 and Rd. The reason for this

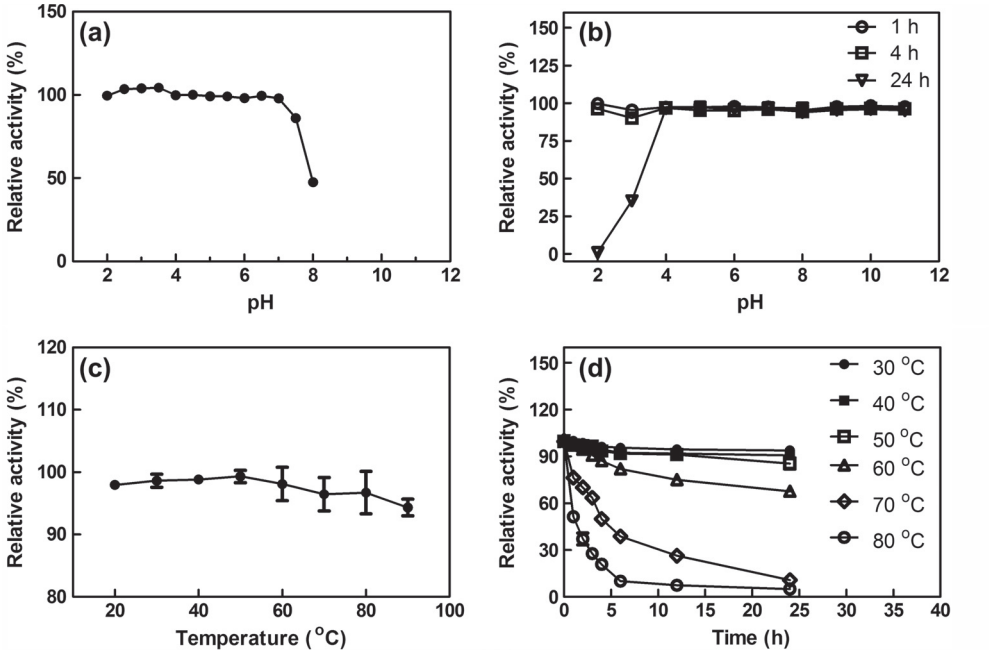

Figure 4. Effect of pH and temperature on activity and stability of GH1: (a) optimal pH, (b) pH stability, (c) optimal temperature, (d) thermostability. Results are presented as means ± standard deviations ($n = 3$).

might be that the spatial conformation of ginsenoside blocked attack of GH1 on the β-(1→2)-glycosidic linkage. In our previous report, the crude enzyme mixture of *P. oxalicum* was used to transform protopanaxadiol-type ginsenosides, the transformation products included Ginsenoside Rd, Compound O, Compound Y, Ginsenoside F2, Ginsenoside Mb, Ginsenoside Mc, and Compound K (Gao et al. 2013). Biotransformation of ginsenosides by the crude enzyme mixture would give a series of minor ginsenosides but is not suitable for ginsenoside Rd preparation since multiple products would reduce the yield of ginsenoside Rd and increase purification complexity.

Time course of hydrolysis of ginsenosides catalyzed by GH1

The transformed products of ginsenosides Rb1, Rb2, and Rc produced by the action of GH1 were analyzed by HPLC. The results showed that the final product of the hydrolysis of ginsenoside Rb1 by GH1 was ginsenoside Rd. After 2 h incubation, most ginsenoside Rb1 was converted into Rd. No other ginsenoside product was observed even when the incubation time was prolonged up to 24 h (Figure 5), indicating that GH1 could only remove the terminal glucose unit at the C-20 site of Rb1, without hydrolyzing other sugar linkages. In contrast, ginsenosides Rb2, Rc, and Rd were not converted by GH1 after 24 h incubation. The transformed product was purified by HPLC and then characterized by ^{13}C-NMR spectroscopy. Comparing the ^{13}C-NMR spectral signals of the substrate ginsenoside Rb1 and bio-transformed product (Table IV), it was noticed that the C-5, C-6 of 20-inner-glucosidic residue in ginsenoside Rb1 shifted from δ76.60 to δ78.19 and δ71.79 to δ71.40, respectively, which confirmed that the 20-outer-glucosidic residue of substrate Rb1 was removed (Zhao et al. 2009a; Cho et al. 2010).

Production of ginsenoside Rd from ginsenoside Rb1 by GH1

The production of ginsenoside Rd from ginsenoside Rb1 by GH1 was optimized and the biotransformation was maximal at pH 3.5 and 55°C (Figure 6a

Table II. The effects of metal ions and reagents on the activity of GH1.

Metal ions or reagents	None	Na^+	K^+	Ca^{2+}	Mg^{2+}	Mn^{2+}	Cu^{2+}	Zn^{2+}	Ba^{2+}	Hg^{2+}	Co^{2+}	SDS	EDTA
Relative activity (%)[a]	100 ± 0.00	96.65 ± 0.65	95.47 ± 2.32	98.56 ± 2.60	100.85 ± 1.76	89.63 ± 1.86	88.85 ± 4.08	98.43 ± 2.97	95.87 ± 3.06	104.66 ± 6.03	95.41 ± 2.04	40.88 ± 6.03	24.34 ± 8.82

[a]The relative activity was expressed with respect to control without divalent metal ions.

Table III. Relative activity of GH1 on different substrates.[a]

*p*NP-substrate	Relative activity (%)[b]	Disaccharide	Relative activity (%)[b]	Ginsenoside	Relative activity (%)[b]
*p*NP-β-D-glucopyranoside	100 ± 0.00	Cellobiose	100.0 ± 0.00	Ginsenoside Rb_1	45.82 ± 3.51
*p*NP-β-D-galactopyranoside	0.16 ± 0.01	Gentiobiose	143.89 ± 0.85	Ginsenoside Rb_2	0 ± 0.00
*p*NP-β-D-mannopyranoside	1.02 ± 0.21	Sophorose	576.57 ± 13.08	Ginsenoside Rc	0 ± 0.00
*p*NP-α-D-glucopyranoside	0.63 ± 0.11	Sucrose	30.36 ± 0.85	Ginsenoside Rd	0 ± 0.00
*p*NP-α-D-galactopyranoside	0.93 ± 0.07	Trehalose	33.00 ± 0.57		
*p*NP-α-D-mannopyranoside	0.41 ± 0.00	Lactose	2.64 ± 0.57		
Methyl-α-D-glucopyranoside	3.89 ± 0.85	Maltose	16.17 ± 3.32		
Methyl-α-D-mannopyranoside	2.67 ± 0.28	CMC	25.08 ± 4.53		

[a]Absorption caused by released *p*-nitrophenyl or reducing sugars (DNS method) was measured at 405 or 520 nm. The hydrolysis product of ginsenosides was measured by HPLC. The relative activities against pNPG or cellobiose were taken as controls (100%), respectively.
[b]Results are presented as means ± standard deviations ($n = 3$).

and b). For ginsenoside Rd production, the pH of the reaction was set at 4.5 due to the instability of the enzyme below pH 4.0. The optimal enzyme concentration was 0.25 U/ml, low enzyme concentrations would slow the transformation reaction while high enzyme concentrations could waste enzyme (Figure 6c). Under the optimal conditions (pH 4.5, 55°C, 0.25 U/ml GH1), about 75 mg Rd was obtained in 60 min from 100 mg ginsenoside Rb1, corresponding to a productivity of 1.25 mg/ml/h (Figure 6d). In our previous work, the crude enzyme solution from *P. oxalicum* was used to convert ginsenoside Rb1 to ginsenoside Rd. Since ginsenoside Rd could be further converted to ginsenoside F2 then the final product Compound K by the crude enzyme solution (Gao et al. 2013), the productivity of ginsenoside Rd was therefore relatively low, about 0.0825 mg/ml/h. The productivity of ginsenoside Rd by GH1 was 75% and 15.2 fold higher than that obtained from the crude enzyme solution.

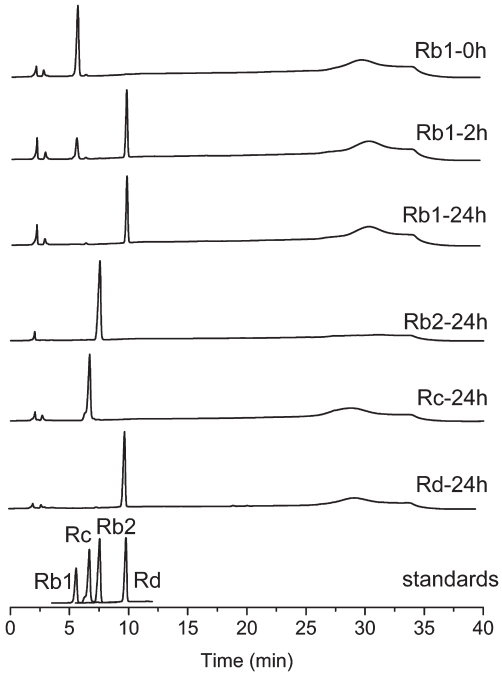

Figure 5. Hydrolysis of ginsenoside Rb1, Rb2, Rc, and Rd by GH1.

Discussion

Minor ginsenosides are regarded as potential antitumor, anti-inflammatory, and immune-enhancing molecules (Kim et al. 2003; Sun et al. 2006; Park et al. 2009; Dong et al. 2011). Preparation of minor ginsenosides with a simple procedure, high productivity, and repeatable operation is of great importance for their application. Biotransformation is designed for the preparation of bioactive minor ginsenosides, in which β-glucosidase plays a significant role. In our previous study, we reported the isolation of a ginsenoside-hydrolyzing strain *P. oxalicum* from soil, which could convert protopanaxadiol-type ginsenosides to active minor ginsenosides (Gao et al. 2013). Using this newly isolated strain, we have obtained a new purified β-D-glucosidase, GH1, which could transform ginsenoside Rb1 to produce ginsenoside Rd. Besides GH1, there were two glucosidase activity peaks eluted from a phenyl sepharose CL-4B column, GH2, and GH3. TLC and HPLC results indicated that GH2 and GH3 could further transform ginsenoside Rd to give other minor ginsenosides (data not shown). Therefore, we deduced that GH1, GH2, and GH3 synergistically catalyzed protopanaxadiol-type ginsenoside Rb1, Rb2, and Rc.

A β-glucosidase was purified from the autolysates of *P. oxalicum*. The enzyme possessed a Mw of 110 kDa on SDS-PAGE and a *p*I of 4.0, and the active conditions were established at pH 5.5 and 55°C (Copa-Patiño et al. 1990). Comparing GH1 and the reported enzyme, we found that β-glucosidases

Table IV. ^{13}C-NMR spectral assignments of ginsenoside Rb1 and its transformed product by GH1.

Sugar moiety	Rb1	Transformed product	Sugar moiety	Rb1	Transformed product
3-O-inner-Glc			20-O-inner-Glc		
C1	104.77	104.77	C1	98.40	98.57
C2	81.36	81.36	C2	75.43	75.62
C3	77.97	77.96	C3	77.97	78.52
C4	71.85	71.85	C4	72.03	72.24
C5	78.22	78.61	C5	76.60	78.19
C6	63.11	62.80	C6	71.79	71.40
3-O-outer-Glc			20-O-outer-Glc		
C1	105.68	105.67	C1	105.27	
C2	77.06	76.60	C2	75.59	
C3	78.81	78.81	C3	78.22	
C4	71.96	71.85	C4	72.03	
C5	77.06	77.96	C5	78.22	
C6	63.41	63.10	C6	61.89	

from *P. oxalicum* had similar acidic *p*I values (from 4.0 to 4.2) and substrate specificities on *p*-nitrophenyl compounds. However, there are also some differences, especially in their stability and selectivity. GH1 showed better stability to pH, metal ions, and detergent, SDS. The good pH and temperature stability of GH1 is of great importance for the ginsenoside biotransformation, which is usually carried out under acidic pH conditions and high temperatures. The excellent stability of GH1 would be useful for its application in ginsenoside biotransformation.

Besides, GH1 has absolute specificity for the β-(1→6)-glycosidic linkage in ginsenoside Rb1, without hydrolyzing ginsenoside Rb2, Rc, and Rd. We have previously purified two β-glucosidases G-I and G-II from *Cladosporium fluvum*, and they could transform ginsenoside Rb1 to Rd as well (Zhao et al. 2009b; Gao et al. 2010). The specificity of GH1 was similar with that of G-I and G-II. However, the transformation efficiency of GH1 was much higher than that of G-I and G-II, indicating that GH1 has more potential in ginsenoside Rd preparation.

Previously, the production of pharmaceutical active ginsenoside Rd from ginsenoside Rb1 has been performed with enzymes, such as β-D-glucosidase from the China white jade snail, cellulase from

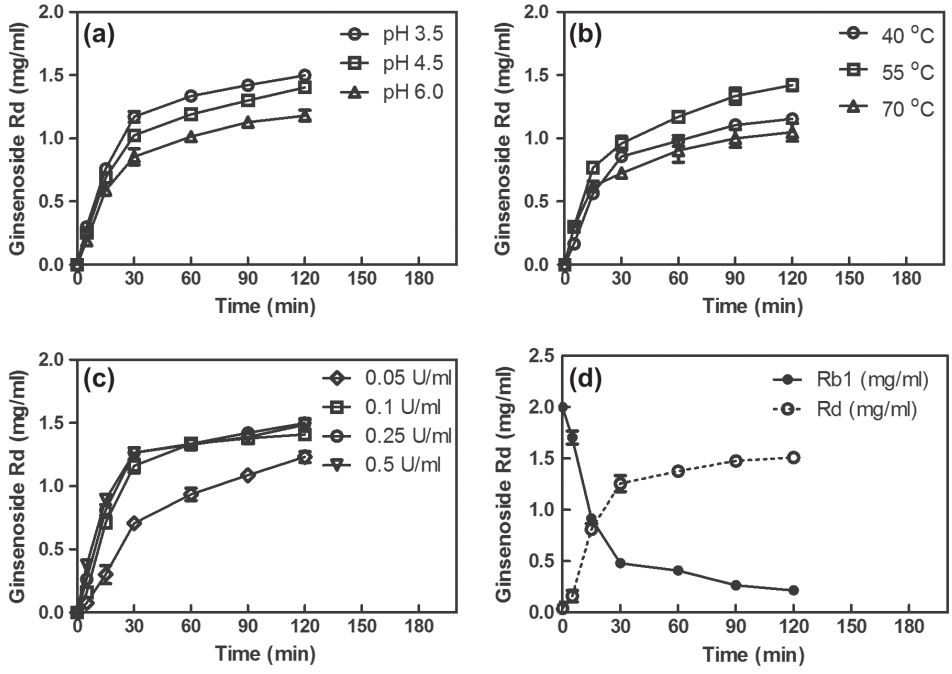

Figure 6. Ginsenoside Rd production from ginsenoside Rb1 by GH1: pH (a), temperature (b), enzyme concentration (c) was optimized, respectively. Under optimal conditions, ginsenoside Rb1 was transformed to Rd (d).

Trichoderma viride and β-D-glucosidases from *C. fluvum* (Hu et al. 2007; Ko et al. 2007; Zhao et al 2009). Compared with these enzymes, GH1 has significant advantages due to its easier preparation, higher specificity and yield, and better stability. GH1 will be further studied to lay a foundation for the application in Rd preparation.

Declaration of interest: The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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