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A novel ginsenoside Rb_1-hydrolyzing β-D-glucosidase from *Cladosporium fulvum*

Xuesong Zhao [a,b], Ling Gao [a], Juan Wang [a], Hongtao Bi [a], Juan Gao [a], Xiuli Du [a], Yifa Zhou [a,*], Guihua Tai [a,*]

[a] *Laboratory of Molecular Epigenetics of MOE, School of Life Sciences, Northeast Normal University, Changchun 130024, PR China*
[b] *Fuxin Advanced Polytechnic College, Fuxin 123000, PR China*

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ABSTRACT

A novel β-glucosidase (G-II) was purified to homogeneity from a culture filtrate of the phytopathogenic fungus *Cladosporium fulvum* (*syn. Fulvia fulva*). G-II specifically cleaved the β-(1 → 6)-glucosidic linkage at the C-20 site of ginsenoside Rb_1 to produce ginsenoside Rd, but did not hydrolyze the other β-D-glucosidic linkages in protopanaxadiol-type ginsenosides. In specificity tests, G-II was active against *p*NPG and disaccharides such as cellobiose and gentiobiose, but exhibited very low activities against other aryl-glycosides and methyl-α-glycosides. G-II consisted of two identical subunits with a native molecular mass of 180 kDa and a p*I* of 4.4. The optimal pH of G-II was pH 5.5, and the enzyme was highly stable over a range of pH 5.0–11.0. The optimal temperature was 45 °C, and the enzyme became unstable at temperatures above 40 °C. The K_m and V_{max} values against *p*NPG were 0.19 mM and 57.7 μmol/(min mg), respectively. The enzyme was inhibited by Zn^{2+}, Cu^{2+} (over 50 mM) and SDS (250 mM). However, the inhibition by SDS was partially reversed by 10 mM dithiothreitol. Three oligopeptide fragments obtained after enzymatic digestion of G-II were sequenced by nanoESI-MS/MS. The amino acid sequence homology analysis showed that G-II possessed significant homology with the family 3 β-glucosidases.

1. Introduction

Ginseng, the root of *Panax ginseng* C.A. Meyer (Araliaceae), has been used as a traditional medicine in oriental countries for over 2000 years. The major active components of ginseng are triterpenoidal dammarane glycosides, namely ginsenosides. Up to now, more than 40 ginsenosides have been isolated and characterized from various ginseng species [1]. Some ginsenosides have a higher content in ginseng, such as ginsenosides Rb_1, Rb_2 and Rc, termed major ginsenosides, while others are present in ginseng at low levels, such as ginsenoside Rd, Rg_3 and Rh_2, termed minor ginsenosides. In recent years, it was found that the minor ginsenosides had remarkable pharmacological activity and might be more potential drug candidates. Ginsenoside Rd, a protopanaxadiol-type ginsenoside, is one of these compounds. Previous studies have shown that ginsenoside Rd can protect neurons from neurotoxic chemicals [2], enhance the differentiation of neural stem cells [3] and prevent the contraction of blood vessels [4]. However, for the preparation of Rd in large amounts, isolating it from ginseng is very difficult because of its low content, usually 4–11% of the total ginsenosides in a ginseng sample [5], and chemically synthesizing it from simple materials is currently impossible. The most promising method is transformation from compounds structurally related to Rd. Ginsenoside Rb_1, another protopanaxadiol-type ginsenoside, has one more sugar residue at the C-20 position than does Rd, and has a higher content, usually composing more than 20% of the total ginsenosides in a ginseng sample [5]. It can be transformed into ginsenoside Rd by hydrolyzing the β-(1 → 6)-glucosidic linkage at the C-20 position of Rb_1 (Fig. 1). Although the transformation can be carried out by chemical methods like acid or alkaline hydrolysis, enzymatic conversion is considered the most promising method due to its high selectivity, mild reaction conditions and environmental compatibility. Accordingly, β-glucosidase is regarded as the most useful enzyme for the biotransformation of ginsenoside Rb_1 into Rd. However, in this biotransformation process, it is difficult to terminate the hydrolysis of ginsenoside Rb_1 at Rd because Rd can be hydrolyzed further to F_2 and compound K (C-K), or to Rg_3 and Rh_2 (Fig. 1) [6–10]. Therefore, it is very important to screen the Rb_1-hydrolyzing β-D-glucosidases with good regio-selectivity.

One β-glucosidase that hydrolyzes ginsenoside Rb_1 to terminate at ginsenoside Rd has been purified and characterized from the China white jade snail [11]. However, among plant, animal and microbial β-glucosidases, a microbial β-glucosidase could be advantageous over its animal/plant counterparts because it could be produced by fermentation at large scales, and would have good reproducibility, low cost and a low contamination rate. Therefore, a

* Corresponding authors. Tel.: +86 431 85098212; fax: +86 431 85098212.
E-mail addresses: zhouyf383@nenu.edu.cn (Y. Zhou), taigh477@nenu.edu.cn (G. Tai).

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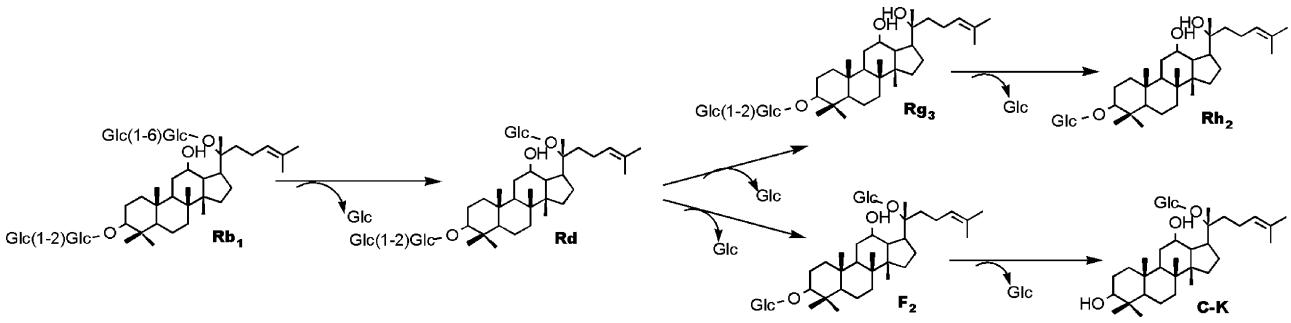

Fig. 1. Transformation pathway of ginsenoside Rb_1.

microbial β-glucosidase is the most desirable because of its industrial application potential. This motivated us to screen novel β-glucosidases from microorganisms with high specificity towards the outer glucose residue at the C-20 of ginsenoside Rb_1. In the present paper, an extracellular β-glucosidase, specifically converting Rb_1 to terminate at Rd, was purified from the tomato pathogen *Cladosporium fulvum*. Furthermore, the properties of this purified β-glucosidase were characterized.

2. Materials and methods

2.1. Materials

p-Nitrophenyl-β-D-glucopyranoside (*p*NPG), *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, methyl-α-D-mannopyranoside, sophorose and D-(+)-trehalose were purchased from Sigma (St. Louis, MO, USA). β-Gentiobiose and D-(+)-cellobiose were from Fluka AD (Switzerland). Ginsenoside Rb_1, Rb_2, Rc and Rd were obtained from Chengdu Mansite Biotechnology Co. Ltd., China. Superose 6 10/300 GL columns, Mono Q HR 5/5 columns and Phenyl-sepharose CL-4B gels were from Amersham Pharmacia. Bio-Scale CHT20-1 columns were from Bio-Rad Laboratories. DEAE-cellulose matrices and Sepharose CL-6B gels were the products of Shanghai Hengxin Co. Ltd., China and Beijing Dingguo Biotechnology Co. Ltd., China, respectively. Shim-pack PREP-ODS (H) Kit columns including a analytical column (4.6 mm × 250 mm, 5 μm) and a preparative column (20 mm × 250 mm, 5 μm) and the HPLC system consisting of two LC-10ATvp pumps and a SPD-10Avp detector were from Shimadzu, Japan. All other reagents were of analytical grade.

2.2. Microorganism and culture conditions

The tomato pathogen *C. fulvum* was obtained from Jilin Academy of Agricultural Sciences in Changchun, China. The strain was isolated from tomato locally and maintained on V8 juice agar medium (per liter: 200 ml of V8 juice, 2 g of $CaCO_3$, 20 g of agar). The spores were collected from 8-day-old cultures on V8 juice agar medium kept at 25 °C in the dark and were incubated in V8 juice liquid medium (per liter: 200 ml of V8 juice, 2 g of $CaCO_3$) at 28 °C with shaking (130 rpm) for 84 h. The culture broth was filtered through absorbent gauze and centrifuged at 10,000 × *g* for 20 min. The supernatant was used as crude enzyme preparation.

2.3. Purification of β-glucosidase from C. fulvum

All purification steps were carried out at room temperature unless otherwise indicated. The purification was monitored by measuring the absorbance at 280 nm and the β-glucosidase activity of each fraction.

One hundred and twenty milliliters (bed volume) of DEAE-cellulose were added to 6 l of crude enzyme preparation. The gel slurry was rotated overnight at 4 °C and subsequently transferred to an empty column (2.8 cm × 25 cm). The unbound material flowed through the column during column packing. The bound materials were eluted stepwise with 240 ml of 0.0 M, 0.25 M, 0.50 M, 0.75 M and 1.0 M NaCl in buffer A (25 mM acetate buffer, pH 5.0) at a flow rate of 3 ml/min. The fractions eluted by 0.25 M and 0.50 M NaCl showed enzyme activity and were pooled as indicated (Fig. 2A). The enzymes in these two pools were referred to as G-I (for glucosidase I) and G-II (for glucosidase II), respectively. G-II was further purified in this paper, as described below. It was precipitated by ammonium sulphate (30–80%) at 0 °C, then dissolved in buffer A and applied onto a Sepharose CL-6B column (3.0 cm × 90 cm). The column was eluted with 0.15 M NaCl in buffer A at a flow rate of 0.15 ml/min (Fig. 2B). The fractions with high enzyme activity (corresponding to 340–410 ml) were combined, adjusted to 1.0 M $(NH_4)_2SO_4$ and applied onto a Phenyl-sepharose CL-4B column (1.5 cm × 10.2 cm) pre-equilibrated with buffer B (buffer A containing 1.0 M $(NH_4)_2SO_4$ and 0.15 M NaCl). The column was eluted first with 36 ml buffer B, then with a 180-ml linear gradient from 100% buffer B to 100% buffer A at a flow rate of 1.0 ml/min (Fig. 2C). The fractions corresponding to 150–192 ml were combined, dialyzed against buffer C (20 mM Tris–HCl buffer, pH 7.5) and applied onto a Mono Q HR 5/5 column. The column was eluted at 1 ml/min with 0.3 M NaCl in buffer C for 15 min followed by a linear gradient of 0.3–0.5 M NaCl in buffer C for 46 min (Fig. 2D). The fractions collected between 31 min and 34 min were combined, dialyzed against 0.01 M phosphate buffer, pH 6.8, and then applied to a Bio-Scale CHT20-1 column (HAC). The column was eluted with the same buffer at 1 ml/min for 30 min, followed by a linear gradient of 0.01–0.5 M phosphate buffer, pH 6.8, for 120 min (Fig. 2E). The fractions with enzyme activity (corresponding to 48–50 min) were recovered.

2.4. Enzyme assays

β-Glucosidase activity was measured with *p*NPG as a substrate [8,12]. The reaction mixture, consisting of 10 mM *p*NPG, 25 mM acetate buffer (pH 5.0) and appropriately diluted enzyme solution, was incubated at 37 °C for 30 min. The reaction was then terminated by the addition of 0.25 M NaOH and the absorption caused by the released *p*-nitrophenyl (*p*NP) was measured at 405 nm. Activities against other *p*-nitrophenyl glycosides were measured in the same way. Activities against disaccharides, methyl-α-glycoside and carboxymethyl cellulose (CMC) were assayed with dinitrosalicylic acid reagent at 520 nm for the determination of reducing sugars [13]. The activities on the hydrolysis of ginsenoside were measured by HPLC. The reaction mixture, consisting of 10 mM ginsenoside, 25 mM acetate buffer (pH 5.0) and appropriately diluted enzyme solution, was incubated at 37 °C for 30 min. Then the reaction was stopped by adding equal volume of *n*-butanol. The *n*-butanol phase was evaporated to dryness under vacuum and subsequently applied on HPLC. The column was eluted with a linear gradient of acetonitrile and water at a ratio of 35:65 (v/v)–50:50 (v/v) within 0–15 min, monitored by the absorbance at 203 nm, eluting rate 1.0 ml/min. The amount of product was calculated according to its 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 Rd under assay conditions.

Time course of hydrolysis of ginsenoside was determined as follows. The mixture of ginsenoside (final concentration 2 mM) and G-II in 25 mM acetate buffer (pH 5.5) was incubated at 37 °C. Aliquots were withdrawn at suitable time intervals and the reaction was terminated by adding equal volume of *n*-butanol. The product was detected by HPLC as mentioned above.

2.5. Purity, molecular weight and isoelectric point determination

The purity and molecular weight of G-II were estimated by both gel filtration chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Gel filtration chromatography was performed on a Superose 6 HR 10/300 column pre-calibrated with a molecular weight marker kit (MW-GF-1000 from Sigma). One hundred microliters of sample was injected and eluted at 0.3 ml/min with 0.15 M NaCl in buffer A. The eluate was monitored by the absorbance at 280 nm and collected at 0.6 ml per fraction. The fractions were assayed for their β-glucosidase activities as described above. SDS-PAGE was performed according to Laemmli [14] on 8% resolving gel. The gel was stained by 0.25% Coomassie Brilliant Blue G-250. The molecular weight was estimated using Protein Molecular Weight Marker from Amersham Biosciences. The isoelectric point (p*I*) of G-II was determined by IEF-PAGE using precast IEF gel (Ampholine PAGplate pH 3.5–9.5) from Amersham on a Multiphor II Electrophoresis unit (Amersham Biosciences). The gels were silver-stained according to the manufactory's instruction (Amersham). The p*I* was estimated using p*I* Calibration Kit (pH 3.5–9.3) from Amersham Biosciences.

2.6. Protein sequencing

Protein sequencing was performed by in-gel digestion of the proteins and sequencing of the different peptides using nanoESI-MS/MS (Q-TOF2, Micromass, UK) at the National Center of Biomedical Analysis, Chinese Academy of Military Medical Sciences. The obtained peptide sequences were analyzed by BLAST2 (http://www.ebi.ac.uk/Tools/blast2).

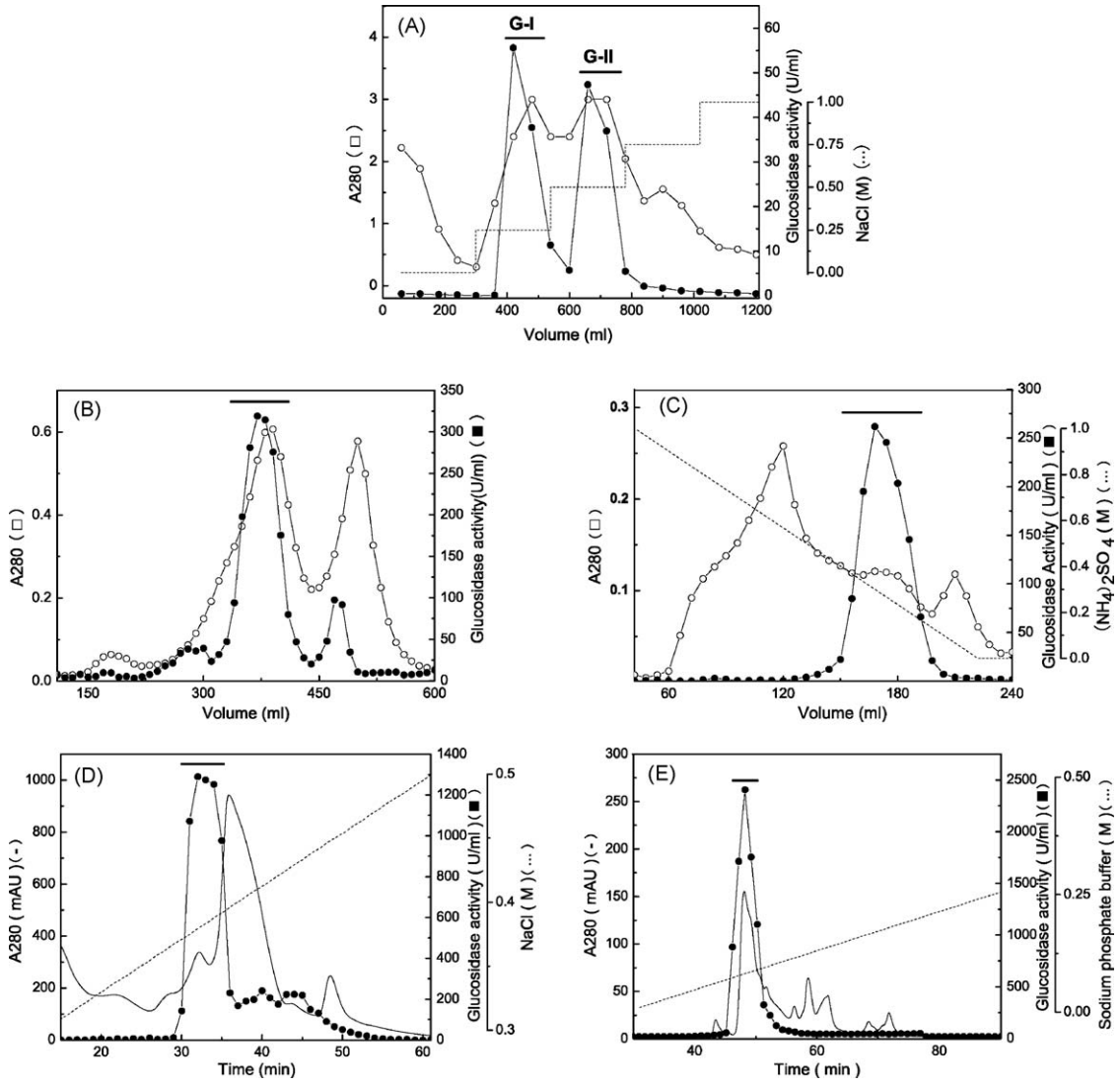

Fig. 2. Purification of β-glucosidase from *C. fulvum*. (A) Ion-exchange chromatography on DEAE-cellulose; (B) gel filtration chromatography on Sepharose CL-6B; (C) hydrophobic interaction chromatography on Phenyl-sepharose CL-4B; (D) ion-exchange chromatography on Mono Q HR 5/5; (E) chromatography on Bio-Scale CHT20-1. (●) represents β-glucosidase activity; (○ or —) represents absorption at 280 nm; (- - -) represents salt gradient. Solid bars represent the fractions pooled for further purification or characterization.

2.7. Assay of optimal pH and pH stability

The optimal pH and pH stability were studied from pH 2.0–12.0 using the following buffer systems: 25 mM Na_2HPO_4–citrate buffer (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 optimal pH was studied by testing the β-glucosidase activity of the purified enzyme at 37 °C at different pHs. The pH stability was determined by preincubating the purified enzyme at 4 °C in the buffers of various pH for 24 h. The residual activities were measured at 37 °C in 25 mM acetate buffer (pH 5.5) as described above [11].

2.8. Assay of optimal temperature and thermal stability

The optimal temperature was determined by measuring the enzyme activities with *p*NPG as a substrate at different temperatures from 20 °C to 60 °C at the optimal pH. The thermal stability of the enzyme was determined by preincubating the enzyme in the temperature range from 20 °C to 60 °C for 2 h and then measuring the residual activities with *p*NPG as a substrate at the optimal temperature [11].

2.9. Effects of metal ions and reagents

The purified enzyme was preincubated at 20 °C with 0.05 M metal ions or 0.25 M reagents in 25 mM acetate buffer (pH 5.5) for 30 min. Enzyme activities were then measured at 37 °C in the presence of the same metal ions or reagents with *p*NPG as a substrate as described above [12].

2.10. Kinetic measurements

The kinetic parameters were determined by measuring the initial reaction velocity at various *p*NPG concentrations ranging from 0.017 mM to 0.33 mM in 25 mM acetate buffer (pH 5.5) at 37 °C as described above. The K_m and V_{max} values were calculated by Lineweaver–Burk plot.

2.11. Isolation and identification of ginsenoside Rb_1-hydrolyzing product

A mixture of ginsenoside Rb_1 (100 mg), 25 mM acetate buffer pH 5.0 (100 ml), and purified enzyme (200 μl) was incubated at 37 °C for 2 h. The reaction was stopped by extraction with *n*-butanol. The *n*-butanol fraction was evaporated to dryness under vacuum and subsequently applied onto a preparative HPLC. The column was eluted with acetonitrile/water (37.5:62.5, v/v) at 5 ml/min and monitored by the absorbance at 203 nm. The fractions containing the product were collected and dried. The product was analyzed by NMR using standard ginsenoside Rd as a reference. The carbon nuclear magnetic resonance (^{13}C NMR) spectrum was obtained on a Bruker Av 600 NMR spectrometer, with CD_3OD as the solvent. The signals and their assignments are as follows: ^{13}C NMR (150 MHz, CD_3OD): δ 16.55 (C-18), 17.00 (C-19), 17.03 (C-29), 17.56 (C-30), 18.24 (C-27), 19.53 (C-6), 23.17 (C-23), 24.54 (C-26), 26.15 (C-16), 27.53 (C-21), 28.67 (C-2), 31.00 (C-15), 31.93 (C-28), 31.31 (C-11), 36.14 (C-7), 36.96 (C-22), 38.20 (C-10), 40.53 (C-1), 40.88 (C-4), 41.29 (C-8), 50.07 (C-13), 51.34 (C-9), 52.79 (C-14), 53.45 (C-17), 57.82 (C-5), 71.85 (C-12), 85.27 (C-20), 91.63 (C-3), 126.31 (C-24), 132.63 (C-25); 3-*O*-glc: δ 62.80 (C-6), 71.85 (C-4), 77.96 (C-3), 78.61 (C-5), 81.36 (C-2), 104.77 (C-1); glc: δ 63.10 (C-6),

71.85 (C-4), 76.60 (C-2), 77.96 (C-5), 78.81 (C-3), 105.67 (C-1); 20-*O*-glc: *δ* 63.40 (C-6), 72.24 (C-4), 75.62 (C-2), 78.19 (C-5), 78.52 (C-3), 98.57 (C-1).

3. Results and discussion

3.1. Production and purification of β-glucosidase G-II

In the screening for ginsenoside Rb_1-hydrolyzing microorganisms, we found that the tomato pathogen *C. fulvum* could convert ginsenoside Rb_1 to Rd and that the enzymes executing this hydrolysis were present in the culture broth. The β-glucosidase activity in the culture broth was measured every 12 h and the maximum activity was reached at 84 h (data not shown). The fermentation was, therefore, stopped at 84 h. The culture filtrates were collected and used as the source for enzyme preparation.

To cope with the large volume of culture filtrates (6 l per preparation), anion-exchange chromatography using a DEAE-cellulose matrix was employed in the first step. Two β-glucosidase-active peaks were observed on the elution profile, one at 0.25 M NaCl and the other at 0.5 NaCl (Fig. 2A). The enzyme eluted with 0.5 M NaCl, referred to as G-II, was further purified and investigated in this paper. The enzyme eluted with 0.5 M NaCl was precipitated by 30–80% ammonium sulphate and subsequently separated by gel filtration chromatography on a preparative Sepharose CL-6B column. Activity measurement revealed two peaks on the column, a major peak at 340–410 ml and a minor peak at 470–480 ml (Fig. 2B). Due to its small value, the minor peak was not further studied. The major peak was recovered and further purified by hydrophobic chromatography on Phenyl-sepharose Cl-4B. The enzyme was eluted with 0.3 M ammonium sulphate on this column, and thus separated from the majority of the contaminating proteins (Fig. 2C). Subsequent purification involved two HPLC columns—a Mono Q HR 5/5 and a Bio-Scale CHT20-1 column. The enzyme was eluted with 0.38 M NaCl on a Mono Q (Fig. 2D) and 0.1 M phosphate on a Bio-Scale CHT20-1 (Fig. 2E) column, thus well separated from the remaining contaminants. After these steps, the purity of the enzyme was examined by SDS-PAGE and high-performance gel filtration chromatography. As shown in Figs. 3 and 4, the enzyme displayed a single band on SDS-PAGE and a single peak on the Superose 6 column, which indicated that the enzyme had been purified to homogeneity. The purification factors and yields in each purification step are summarized in Table 1. The specific activity of the purified enzyme was 23,769 U/mg protein.

3.2. Molecular weight and isoelectric point of G-II

The molecular weight of G-II was estimated to be 97 kDa by SDS-PAGE and 180 kDa by gel filtration chromatography (Fig. 3B and 4). As SDS-PAGE reflects the denatured state and gel filtration reflects the native state, the data above suggest that the native enzyme may consist of two identical subunits. It has been reported by other research groups that β-glucosidases purified from different microorganisms possessed molecular weights ranging from 40 kDa to 440 kDa and a variety of quaternary structures from monomers to octamers [16–19]. G-II is similar to β-glucosidases isolated from *Humicola grisea var. thermoidea*, *Thermomyces lanuginosus*-SSBP and *Sclerotinia sclerotiorum* [19–21] in terms of molecular weight and oligomeric properties. The p*I* of G-II was determined by IEF-PAGE to be 4.4 (Fig. 3A) which is consistent with the report that β-glucosidases from fungi generally have acidic p*I*s [22]. No charge heterogeneity was observed upon the IEF, suggesting only one β-glucosidase to be present in the final preparation.

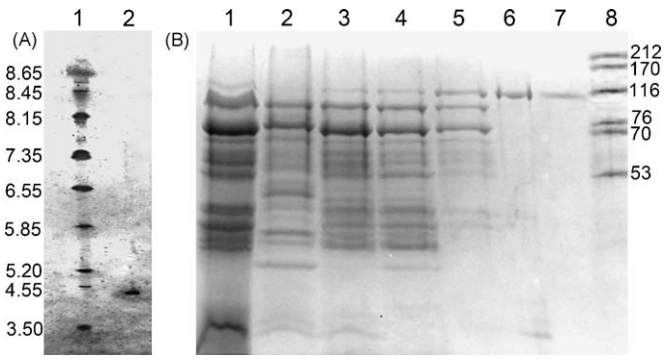

Fig. 3. IEF-PAGE and SDS-PAGE analysis of the G-II. (A) IEF-PAGE on Ampholine PAGplate pH 3.5–9.5. Lane 1, standard p*I* markers; lane 2, purified G-II. (B) SDS-PAGE on 8% resolving gel. Lane 1, crude extract; lane 2, DEAE-cellulose; lane 3, ammonium sulphate precipitation; lane 4, Sepharose CL-6B; lane 5, Phenyl-sepharose CL-4B; lane 6, Mono Q HR 5/5; lane 7, Bio-Scale CHT20-1; lane 8, high molecular weight protein markers.

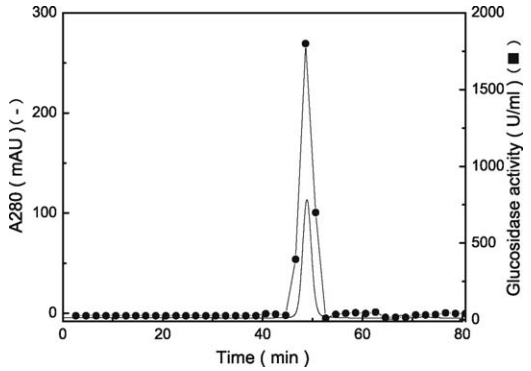

Fig. 4. Gel filtration chromatography on Superose 6 10/300 GL column. (●) and (—) represent β-glucosidase activity and protein (A280), respectively.

3.3. Amino acid sequence analysis

The purified enzyme was digested with trypsin and analyzed with nanoESI-MS/MS. Three peptide sequences (GVDVLLGQGLLAPR, TPFTGEGPSQK and SYGTELLSKPNDGK) were obtained and subjected

Table 1
Purification of β-D-glucosidase from *Cladosporium fulvum*.

Purification steps	Protein[a] (mg)	Total activity (U[b])	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	2050.40	90,170	44	1	100
DEAE-cellulose	168.30	26,964	160	4	30
30–80% $(NH_4)_2SO_4$	19.00	19,945	1,050	24	22
Sepharose CL-6B	7.94	17,378	2,189	50	19
Phenyl-sepharose CL-4B	2.12	7,739	3,650	83	8.6
Mono Q	0.45	6,652	14,782	336	7.4
Bio-Scale CHT20-1	0.26	6,180	23,769	540	6.8

[a] Protein contents were determined according to the method of Bradford [15], using bovine serum albumin as the standard.
[b] One unit (U) of β-D-glucosidase was defined as the amount of enzyme liberating 1 nmol/min of *p*-nitrophenyl.

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                    ├─► peptide 1 ◄─┤ ├─► peptide 2 ◄─┤ ├─► peptide 3 ◄─┤          Accession number
C. Fulvum           ---GVDVLLGQGLLAPRTPFTGEGPSQKSYGTELLSKPNDGK--------           P85517
T. aurantiacus      GNSLVDVLYGRVSPGGKTPFT-WGKTRESYGAPLLTKPNNGKGAPQDDFT 628      Q0ZUL0
N. fischeri         GNSLVDVLYGRVNPSAKTPFT-WGKTRKSYGAPLLSEPNNGNGAPQDDFN 638      A1D451
B. fuckeliana       GNSITDVLYGKVNPAARTPFT-WGPTRESYGTDVLYEPNNGEAAPQLDFT 653      A6SBH0
T. reesei           GNSLVDILYGKQSPG-RTPFT-WGPSLESYGVSVMTTPNNGNGAPQDNFN 633      Q7Z9M5
                        .*:* *:   . :****  * : :***. ::  **:*
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Fig. 5. Multiple sequence alignment of peptide sequences obtained from *C. fulvum* β-glucosidase G-II. The peptide sequences of G-II are aligned with those of *Thermoascus aurantiacus*, *Neosartorya fischeri*, *Botryotinia fuckeliana* and *Trichoderma reesei*. The running total number of amino acids is shown on the right. "*" means that the residues are identical in all sequences in the alignment; ":" means that conserved substitutions are observed. "." means that semi-conserved substitutions are observed.

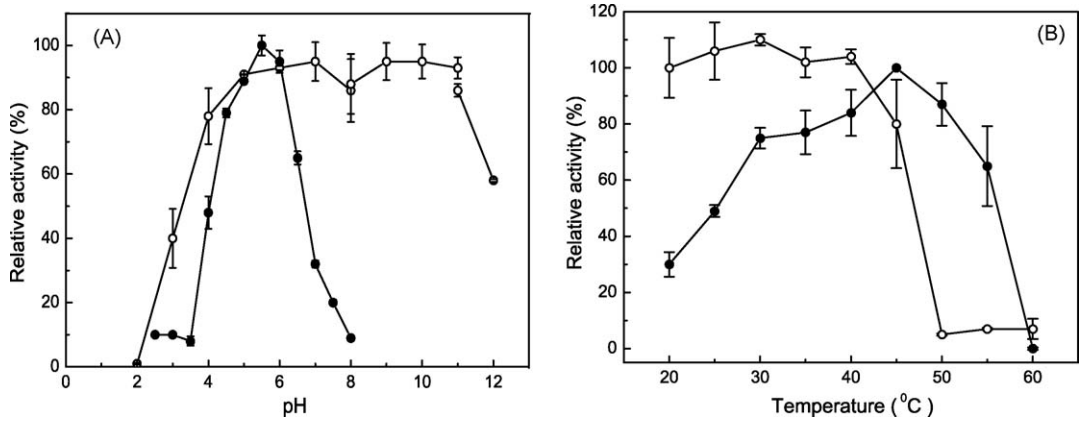

Fig. 6. Effect of pH (A) and temperature (B) on the activity (●) and stability (○) of the β-glucosidase G-II purified from *C. fulvum*. The optimal pH was determined using *p*NPG as a substrate at different pH values at 37 °C. The maximum activity obtained was defined as 100% activity. The pH stability of the enzyme was determined by incubating the enzyme for 24 h at 4 °C in different buffers within the pH range 2–12, and the residual activity was measured at pH 5.5 and 37 °C. The activity of the enzyme before incubation was defined as 100%. The optimal temperature was determined out of various temperatures from 20 °C to 60 °C at optimal pH. Thermal stability of the enzyme was determined by preincubating the enzyme for 2 h at temperatures from 20 °C to 60 °C. The residual activities were then measured at the optimal temperature with *p*NPG as a substrate. The maximum activity observed and the original activity without preincubation were defined as 100%. Results are presented as means ± standard deviations (n = 2).

to the UniProt Knowledgebase (European Bioinformatics Institute) using the WU-BLAST2 network service in a search for proteins that matched the amino acid sequences of G-II, but no sequences were retrieved. G-II may therefore be a novel glycosidase. Compared to all the protein sequences in the UniProt Knowledgebase, the peptide sequences of G-II had high homology to some fungal β-glucosidases or hypothetical proteins in glycoside hydrolase family 3. This suggested that G-II might be a novel member of glycoside hydrolase family 3 (Fig. 5). The protein sequence data reported in this paper will appear in the UniProt Knowledgebase under the accession number P85517.

3.4. Effects of pH and temperature

The effects of pH and temperature on enzyme activity were studied with *p*NPG as a substrate. The optimal pH for the purified G-II was 5.5 (Fig. 6A). After preincubation at 4 °C for 24 h within the pH range 5.0–11.0, the enzyme retained more than 85% of its initial activity, which showed that the enzyme was stable over a wide pH range. The effects of temperature on enzyme activity were studied at the optimal pH (5.5). As shown in Fig. 6B, the optimal temperature of G-II was 45 °C. The enzyme was stable at temperatures lower than 40 °C. After incubation for 2 h at temperatures below 40 °C, it maintained almost full activity. However, the activity decreased rapidly above 45 °C, and the enzyme was completely inactivated at 50 °C after 2 h incubation.

3.5. Effects of metal ions and reagents

The effects of metal ions and reagents on the activity of G-II are shown in Table 2. Enzyme activity was strongly inhibited by Cu^{2+} and Zn^{2+}, and slightly inhibited by Ca^{2+}, Ba^{2+} and Mg^{2+}. In contrast, Na^{+}, K^{+} and Mn^{2+} slightly activated G-II. EDTA, a chelating agent, inhibited the activity to a moderate extent, suggesting that divalent cations were required for the enzyme's function. A possible candidate for such a cation is Mn^{2+}. Other cations including Cu^{2+}, Zn^{2+}, Ca^{2+}, Ba^{2+} and Mg^{2+} might not be involved, as these ions were shown to inhibit the enzyme. SDS at 0.25 M strongly inhibited G-II's activity, as it did with many enzymes. This inhibition could be partially reversed by 10 mM dithiothreitol (DTT), which suggested that a sulphydryl group was involved in the catalytic activity of G-II.

3.6. Kinetic parameters

Kinetic parameters were determined using different *p*NPG concentrations (Fig. 7). The purified enzyme obeyed the Michaelis–

Table 2
The effects of metal ions and reagents on the activity of β-glucosidase G-II from *C. fulvum*.

Metal ions or reagents	Relative activity (%)
None	100 ± 0.00
KCl	104 ± 3.24
NaCl	103 ± 0.68
$CaCl_2$	84 ± 7.32
$BaCl_2$	80 ± 5.98
$MnCl_2$	101 ± 4.36
$MgSO_4$	81 ± 7.56
$CuCl_2$	21 ± 9.46
$ZnCl_2$	26 ± 5.78
SDS	5 ± 0.93
EDTA	59 ± 7.96
SDS + DTT	53 ± 2.54

The relative activity was determined by preincubating the purified enzyme with 0.05 M metal ions or 0.25 M reagents in 25 mM acetate buffer (pH 5.5) at 20 °C for 30 min, then measuring β-glucosidase activity at 37 °C in the presence of the same metal ions and reagents with *p*NPG as a substrate. The activity assayed in the absence of cations or reagents was defined as 100%. Results are presented as means ± standard deviations (n = 2).

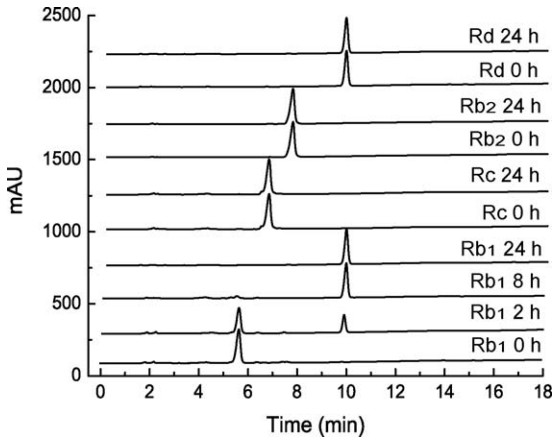

Fig. 7. HPLC analysis of the hydrolysis of ginsenoside by the purified β-glucosidase G-II. The reaction mixture (0.8 ml) containing 2.0 mM of ginsenoside, 25 mM acetate buffer (pH 5.5) and 4.3 U of enzyme was incubated at 37 °C for up to 24 h. Aliquots were withdrawn at suitable time intervals and the reaction was terminated by adding water-saturated *n*-butanol. The *n*-butanol fraction was subjected to HPLC analysis as described in Section 2.

Table 3
Relative activity of β-glucosidase G-II from *C. fulvum*.

Substrate	Relative activity (%)
p-Nitrophenyl-β-D-glucopyranoside	100 ± 0.00
p-Nitrophenyl-β-D-galactopyranoside	2.4 ± 1.20
p-Nitrophenyl-β-D-mannopyranoside	0.3 ± 0.14
p-Nitrophenyl-α-D-glucopyranoside	0.3 ± 0.57
p-Nitrophenyl-α-D-galactopyranoside	0.3 ± 0.14
p-Nitrophenyl-α-D-mannopyranoside	0.6 ± 0.14
Methyl-α-D-glucopyranoside	2.6 ± 0.42
Methyl-α-D-mannopyranoside	3.8 ± 0.66
Ginsenoside Rb_1	58.3 ± 0.18
Ginsenoside Rb_2	0 ± 0.00
Ginsenoside Rc	0 ± 0.00
Ginsenoside Rd	0 ± 0.00
Cellobiose	100.0 ± 0.00
Gentiobiose	97.3 ± 7.58
Sophorose	97.1 ± 4.13
Sucrose	27.9 ± 5.21
Trehalose	5.8 ± 0.57
CMC	13.6 ± 0.14

β-Glucosidase activity was assayed by mixing 5.0 U of the purified enzyme and 0.5 ml of 25 mM acetate buffer (pH 5.0) containing each substrate at 37 °C for 30 min. Absorption caused by the released *p*-nitrophenyl (*p*NP) or reducing sugars (DNS method) was measured at the corresponding wavelength of the initial substrate. The converting product of ginsenoside was measured by HPLC. The relative activities against *p*NPG or cellobiose, respectively, were taken as controls (100%). Results are presented as means ± standard deviations ($n = 3$).

Menten equations over the substrate concentration ranges tested in this paper. The K_m and V_{max} values were calculated by the Lineweaver–Burk method to be 0.19 mM and 57.7 μmol/(min mg), respectively.

3.7. Substrate specificity

The substrate specificities of G-II were studied using different ginsenosides, various *p*-nitrophenyl glycosides, methyl-α-glycosides, disaccharides and CMC (Table 3). When using different ginsenosides as substrates, G-II exhibited high hydrolysis activity on ginsenoside Rb_1 and no activities on Rb_2, Rc and Rd. These ginsenosides have identical protopanaxadiol cores and a disaccharide (glucose-β-(1 → 2)-glucose-β-) substitution at C-3. The difference among them was the sugar residues substituted at C-20, which was glucose-β-(1 → 6)-glucose-β- for Rb_1, arabinose(*p*)-α-(1 → 6)-glucose-β- for Rb_2, arabinose(*f*)-α-(1 → 6)-glucose-β- for Rc and glucose-β- for Rd. Therefore, G-II was highly active on the linkage of glucose-β-(1 → 6)-glucose in these ginsenosides, but did not hydrolyze other glycosidic linkages. As shown in Fig. 7, the time course assay by HPLC analysis also showed that Rb_1 was converted to the sole product. After 2 h incubation with G-II, 34% of the Rb_1 was transformed to Rd; after 24 h, all Rb_1 was converted, but no other ginsenosides appeared. Ginsenoside Rb_2, Rc and Rd were not converted after 24 h incubation. To confirm the product, it was purified using a preparative Shim-pack PREP-ODS (H) column and characterized by ^{13}C NMR spectroscopy. All signals in the ^{13}C NMR spectrum of the product were identical to those of ginsenoside Rd as published in the literatures [9,10,23].

These results above indicated that G-II was very highly selective for hydrolysis of the β-(1 → 6)-glucosidic linkage at C-20 in protopanaxadiol-type ginsenosides, without any attack on other linkages. Therefore, Rb_1 can be selectively converted by G-II into Rd with the conversion terminating at Rd. The high selectivity is very important for industrial production. Up to now, only one β-glucosidase hydrolyzing ginsenoside Rb_1 to terminate at Rd has been purified and characterized from China white jade snail. However, it is unknown whether this β-glucosidase could hydrolyze the other two protopanaxadiol-type ginsenosides Rb_2 and Rc. Considering the capacity for large-scale production of Rd, G-II, isolated from microorganisms, might hold more potential usage for the industry.

It has always been known that the specificity of β-D-glucosidase varies based on a same glycosidic linkage in different glycosides, oligosaccharides and polysaccharides. In the present tests using various *p*-nitrophenyl glycosides and methyl-α-glycosides as substrates, G-II showed high specificity for β-D-glucoside compared to other *p*-nitrophenyl glycosides (Table 3). If the activity against *p*-nitrophenyl-β-D-glucopyranoside is defined as 100%, the activities against both other *p*-nitrophenyl-β- and *p*-nitrophenyl-α-glycosides, as well as against methyl-α-glycosides, was less than 4%. Among disaccharides, G-II showed high activities against cellulose (glucose-β-(1 → 4)-glucose), gentiobiose (glucose-β-(1 → 6)-glucose) and sophorose (glucose-β-(1 → 2)-glucose). As shown in Table 3, using cellulose as a standard reference, the relative activities against gentiobiose and sophorose were 97.3% and 97.1%, respectively. For other two disaccharides, sucrose (glucose-α, β-(1 → 2)-fructose) and trehalose (glucose-α, α-(1 → 1)-glucose), which are linked by a glycosidic bond formed from two acetyl hydroxyl groups, G-II showed 27.9% and 5.8%, respectively, of its activity against cellobiose. In addition, G-II had 13.6% activity against carboxymethyl cellulose.

G-II selectively hydrolyzed β-(1 → 6)-glucosidic linkage of ginsenoside Rb_1, and did not attack β-(1 → 2)-glucosidic linkage. However, it exhibited high activity on sophorose (β-(1 → 2)-glucosidic linkage). These results were similar to those of ginsenoside Rb_1-hydrolyzing β-D-glucosidases from China white jade snail [11]. The reason of the conflicting results might be that the spatial conformation of Rd blocked the attack of G-II to 3-C, β-(1 → 2)-glucosidic linkage, which resulted in the affinity decreasing between the enzyme and Rd.

In conclusion, we successfully purified a novel ginsenoside Rb_1-hydrolyzing β-D-glucosidase (G-II) from the phytopathogenic fungus *C. fulvum* by a combination of ammonium sulphate precipitation, ion-exchange, gel filtration chromatography, hydrophobic chromatography and hydroxyapatite chromatography. G-II can selectively transform ginsenoside Rb_1 into Rd with a high yield. The amino acid sequence homology analysis showed that G-II had significant homology with β-glucosidases or hypothetical proteins of family 3. It may be a novel member of glycoside hydrolase family 3.

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