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Properties of a novel β-glucosidase from *Fusarium proliferatum* ECU2042 that converts ginsenoside Rg_3 into Rh_2

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ABSTRACT

A novel β-glucosidase from *Fusarium proliferatum* ECU2042 (FPG) was successfully purified to homogeneity with a 506-fold increase in specific activity. The molecular mass of the native purified enzyme (FPG) was estimated to be approximately 78.7 kDa, with two homogeneous subunits of 39.1 kDa, and the p*I* of this enzyme was 4.4, as measured by two-dimensional electrophoresis. The optimal activities of FPG occurred at pH 5.0 and 50 °C, respectively. The enzyme was stable at pH 4.0–6.5 and temperatures below 60 °C, and the deactivation energy (E_d) for FPG was 88.6 kJ mol^{-1}. Moreover, it was interesting to find that although the purified enzyme exhibited a very low activity towards *p*-nitrophenyl β-D-glucoside (pNPG), and almost no activity towards cellobiose, a relatively high activity was observed on ginsenoside Rg_3. The enzyme hydrolyzed the 3-C, β-(1 → 2)-glucoside of ginsenoside Rg_3 to produce ginsenoside Rh_2, but did not sequentially hydrolyze the β-D-glucosidic bond of Rh_2. The K_m and V_{max} values of FPG for ginsenoside Rg_3 were 2.37 mM and 0.568 μmol (h mg protein)$^{-1}$, respectively. In addition, this enzyme also exhibited significant activities towards various alkyl glucosides, aryl glucosides and several natural glycosides.

1. Introduction

Ginseng is one of the most widely used Chinese traditional medicines. Ginseng saponins (ginsenosides) were reported to be one of the most important physiologically active materials with a variety of biological effects in ginseng. Ginsenside Rh_2 showed a suppressing effect on cell growth of various cancer cells, therefore having attracted more and more attentions recently [1]. However, the contents of naturally occurring ginsenoside Rh_2 in red ginseng and wild ginseng are only 10 and 30 ppm, respectively [2]. Compared to the structure of other protopanaxadiol-type saponins with higher contents in the ginseng, ginsenoside Rh_2 has the same aglycone, but different sugar moiety. Therefore, it would be a feasible method to produce ginsenoside Rh_2 by modifying the sugar moiety of those ginsenosides that are major or easily available in ginseng. Some methods have been reported regarding the preparation of ginsenoside Rh_2 in this way [3–8], and the selective hydrolysis of ginsenoside Rg_3 using a specific β-glucosidase is a promising one among them (Scheme 1).

β-Glucosidase (EC 3.2.1.21) is an enzyme that hydrolyzes β-glycosidic bonds between the reducing side of glucose and an aryl or alkyl aglycone or an oligosaccharide. β-Glucosidase occurs ubiquitously in plants, animals, fungi and bacteria [9]. It plays key roles in a variety of fundamental physiological and biotechnological processes depending on the nature and diversity of the glycone or aglycone moiety of their substrates, because significant differences in substrate specificity of β-glucosidases are found even if they come from the same source. However, most commonly seen in fungi are the β-glucosidases with much broader specificity which act equally well on both β-oligoglucosides and aryl or alkyl β-glucosides [10], so fungal β-glucosidases are more likely to act as multi-purpose catalysts.

In our previous report, ginsenoside Rh_2 was prepared by enzymatic hydrolysis of ginsenoside Rg_3 using the cell-free extract of a newly isolated strain, *Fusarium proliferatum* ECU2042 [11], which is currently deposited at China General Microbiological Culture Collection Center with an accession number of CGMCC 1495. However, no further information was available concerning this new enzyme (FPG), since the β-glucosidase content in the total proteins of the wild-type strain was extremely low (<0.2%) and thus very difficult to isolate. In this paper, we report the successful purification (up to 500-fold) of the β-glucosidase (FPG) from this fungal strain as well as the characteristic properties of the purified enzyme, including its specificity to various natural or artificial substrates, in order to provide convenience for further application.

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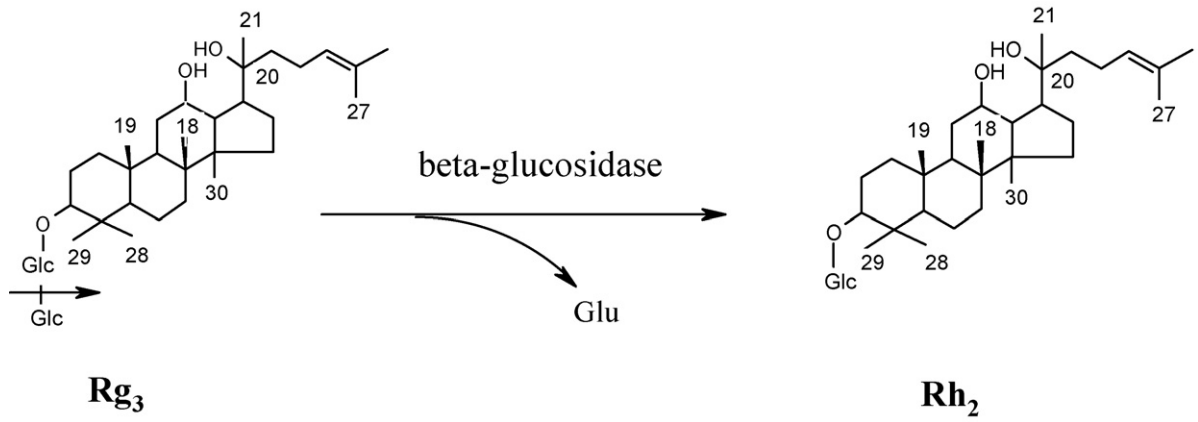

Scheme 1. Enzymatic transformation of ginsenside Rg_3 to Rh_2.

2. Materials and methods

2.1. Materials

Ginsenoside Rg_3, ginsenoside Rh_2 and soy saponins were purchased from Hongjiu Biotech Co. Ltd, Jilin, China. *p*-Nitrophenyl β-D-glucopyranoside (pNPG) and *p*-nitrophenyl β-D-galactopyranoside were obtained from Sigma Chemical Co., USA. Esculin was purchased from Lancaster Co., UK. Various alkyl glucosides were home synthesized by Dr. Lu and other members of our laboratories [12–14]. DEAE-Toyopearl 650M, Butyl-Toyopearl 650M and TSK-gel column were purchased from Tosoh, Tokyo, Japan. All other chemicals were obtained from local suppliers and of reagent grade.

2.2. Microorganism cultivation and crude enzyme extraction

These steps were performed as described previously [11].

2.3. β-Glucosidase activity assay

The activity of FPG on ginsenoside Rg_3 was determined as follows: the standard reaction mixture consisted of 100 μl of enzyme solution, 80 μl of NaAc/HAc buffer (0.5 M, pH 5.0) and 20 μl of ginsenoside Rg_3 solution (5 mg ml^{-1}, in water), giving a final substrate concentration of 0.5 mg ml^{-1}. After incubation (at 50 °C and 160 rpm) for 3 h, the reaction mixture was extracted by 200 μl of *n*-butanol. After centrifugation, the supernatant was directly subjected to HPLC analysis (as described previously [11]) to determine the quantity of ginsenoside Rh_2 generated. One unit of β-glucosidase activity (U) was defined as the amount of enzyme catalyzing the formation of 1.0 nmol ginsenoside Rh_2 per hour under above conditions.

The hydrolytic activities of FPG on pNPG and *p*-nitrophenyl β-D-galactopyranoside were determined by measuring the release of *p*-nitrophenol spectrophotometrically, as described previously [14]. The spontaneous hydrolysis of the substrates without enzyme was subtracted.

The hydrolytic activities of FPG on various glucosidic substrates were determined based on the amount of glucose released from those glucosides. A mixture containing 100 μl of enzyme solution, 80 μl of NaAc/HAc buffer (0.5 M, pH 5.0) and 20 μl of a glucoside solution (in water) was incubated at 50 °C for 24 h, and the glucose released into the mixture was determined spectrophotometrically using a glucose assay kit (purchased from Shenfeng Biotech Co. Ltd, Shanghai, China). Control experiments were performed similarly by omitting either the substrate or the enzyme.

2.4. Purification of F. proliferatum β-glucosidase FPG

All purification operations were performed at 4 °C. The protein concentration was routinely estimated by measuring the absorbance at 280 nm or precisely measured according to Bradford's method using bovine serum albumin as a standard.

The crude enzyme extract was brought to 80% saturation by the addition of solid ammonium sulfate, slowly stirred for 1 h, and centrifuged (10,000 × *g* for 30 min), then the supernatant was loaded onto Butyl-Toyopearl 650M column (Φ1.2 cm × 15 cm, bed volume: 20 ml) equilibrated with Buffer A (20 mM NaAc/HAc buffer, pH 5.0, containing $(NH_4)_2SO_4$ at 60% saturation). The enzyme was first washed with 200 ml of Buffer A and then eluted with a linear gradient of $(NH_4)_2SO_4$ solution (60–30% saturation, 200 ml) at a flow rate of 1 ml min^{-1}. The active fractions were pooled, dialyzed, and then applied to pre-equilibrated DEAE-Toyopearl 650 M column (Φ1.2 cm × 15 cm, bed volume: 20 ml). The adsorbed proteins were eluted stepwisely by NaCl gradients (*i.e.*, 0.06, 0.08 and 0.10 M) in Buffer B (10 mM Tris–HCl buffer, pH 8.0), using 100 ml of the buffer solution for each step and flowing at a rate of 1 ml min^{-1}. The active fractions mainly locating in 0.10 M NaCl eluent were pooled and applied to a TSK-gel filtration column (Φ1.0 cm × 30 cm) connected to a HPLC system. After elution with KPB (100 mM, pH 6.7, with 100 mM Na_2SO_4) at a flow rate of 0.25 ml min^{-1}, the active enzyme fractions were pooled, combined and concentrated by ultrafiltration (the pore diameter of the filter membrance was 10.0 kDa). The concentrated enzyme solution was then subjected to a discontinuous SDS-renaturing-PAGE (SDS-R-PAGE) with activity staining (as described below), and then the black bands of the gel were separately cut and putted into a dialyzer containing Buffer B. The gel slices were electro-eluted at 100 V for 3 h in an electrophoresis apparatus with 25 mM Tris/192 mM glycine buffer. The eluted samples were concentrated and subjected to another round of SDS-R-PAGE, activity staining and electro-elution.

2.5. SDS-renaturing-PAGE and activity staining

SDS-renaturing-PAGE (SDS-R-PAGE) [15] was performed on a 10% separating gel (450 mM Tris–HCl, pH 8.8) and 3.7% stacking gel (60 mM Tris–HCl, pH 6.8) at 4 °C. Different from SDS-PAGE, the samples subjecting to SDS-R-PAGE were mixed with a sample buffer free of β-mercaptoethanol, without incubation at a high tempera-

ture (100 °C) before loading on the gel. After electrophoresis, the gel was incubated for 30 min in 250 ml of a cleaning mixture (Tris base 3.03 g, Triton X-100 12 ml and distilled water 500 ml, pH 6.5) for the removal of SDS. The Triton X-100 was then removed by incubating the gel in distilled water.

For activity staining [16], the gel was first immersed in 0.2 M NaAc/HAc buffer (pH 5.0) for 10 min in order to exchange the buffer system, then incubated in NaAc/HAc buffer containing 0.1% (w/v) esculin and 0.03% (w/v) ferric chloride at 50 °C for 15–60 min. During incubation, a black band corresponding to the β-glucosidase appeared against a transparent background. The staining was stopped by immersing the gel in 10% (w/v) glucose solution.

2.6. Molecular mass determination and two-dimensional electrophoresis

The molecular mass of FPG subunit was estimated by SDS-PAGE using bovine carbonic anhydrase (31.0 kDa), rabbit actin (43.0 kDa), bovine serum albumin (66.2 kDa) and rabbit phosphorylase b (97.4 kDa) as standard proteins. The molecular mass of the native enzyme was determined via HPLC with a TSK-gel column and calculated by comparing with standard proteins (rabbit actin 43.0 kDa, bovine serum albumin 66.2 kDa, rabbit phosphorylase b 97.4 kDa, calmodulin-binding protein 130 kDa, and myosin 200 kDa). All samples were eluted with a solution consisting of 100 mM KPB (pH 6.7) and 100 mM Na_2SO_4 at a flow rate of 0.25 ml min^{-1} and detected at 280 nm.

Two-dimensional electrophoresis (2-DE) was performed as described previously [14].

2.7. Effects of pH and temperature on enzyme activity and stability

The effect of pH on FPG activity was determined by assaying the enzyme at 50 °C with 0.2 M citric acid buffer within the pH ranges of 3.0–7.0. The pH stability was determined by standard enzyme assay using ginsenoside Rg_3 after preincubating the purified enzyme in buffer solutions of different pH at 4 °C for 24 h.

The effect of temperature on enzyme activity was determined by assaying the purified enzyme at temperatures from 30 to 70 °C by standard enzyme assay using ginsenoside Rg_3. Thermal stability was measured by incubating the enzyme in 0.2 M NaAc/HAc buffer (pH 5.0) at temperatures from 30 to 70 °C, and then the remaining activities at different time intervals were determined under standard enzyme assay conditions.

2.8. Effect of metal ion and EDTA on enzyme activity

The effects of various metal ions and EDTA at 10 mM on FPG activity were determined by measuring the enzyme activity with ginsenoside Rg_3 in the presence of metal ion or EDTA under standard enzyme assay conditions. The activity assayed in the absence of metal ion or EDTA was taken as 100%.

2.9. Substrate specificity and kinetic study

Several alkyl or aryl glucosides (10 mM) and natural glycosides (0.5 or 1%, w/v) were tested as substrates. Hydrolysis of ginsenoside Rg_3, pNPG, *p*-nitrophenyl β-D-galactopyranoside and all other substrates were assayed by standard procedures, as described above (Section 2.3).

The effect of ginsenoside Rg_3 concentration (0.5–3.2 mM) on the reaction rate was determined at pH 5.0 and 50 °C. The values of the Michaelis constant (K_m) and the maximum velocity (V_{max}) were determined from Lineweaver–Burk plots.

2.10. Time course of ginsenoside Rg_3 biohydrolysis

The enzyme was incubated with Rg_3 (final concentration of Rg_3: 1 mg ml^{-1}) in 0.2 M NaAc/HAc buffer (pH 5.0) at 50 °C. Aliquots were withdrawn at suitable time intervals and extracted by same volumes of *n*-butanol. After centrifugation, the reaction product was detected by HPLC analysis as described previously [11].

3. Results and discussion

3.1. Purification and molecular properties of FPG

The β-glucosidase from crude enzyme extract of *F. proliferatum* ECU2042 (FPG) was purified as described in Section 2.4. When the concentration of $(NH_4)_2SO_4$ reached 80%, only a little fraction of FPG was precipitated, and over 90% of the enzyme was kept in the supernatant, exhibiting twofold activity possibly because some inhibitors were eliminated by $(NH_4)_2SO_4$ precipitation. The supernatant was purified by the combination of Butyl-Toyopearl 650M, DEAE-Toyopearl 650M, TSK-gel column and two rounds of SDS-R-PAGE. Finally, FPG was purified by approximately 506-fold, with a total activity yield of 1.74% relative to the crude enzyme. The specific activity of the purified enzyme was 2458 U (mg protein)$^{-1}$. The purification results were summarized in Table 1. One single band produced by activity staining of the renatured gel (lane 4 in Fig. 1) was coincident with the one stained with silver (lane 3 in Fig. 1). Moreover, the protein collected from the active band was also demonstrated a single band by SDS-PAGE stained with silver (lane 2 in Fig. 1), confirming that the active component obtained by SDS-R-PAGE (2) was a pure protein.

Molecular mass of FPG, estimated, respectively, by SDS-PAGE and TSK-gel column, were 39.1 and 78.7 kDa, suggesting that the purified enzyme was comprised of two identical subunits. This FPG is different from other ginsenoside β-glucosidases reported so far, whose molecular masses were 34, 320, 59, 31, 460, 230, 220 and 80 kDa, respectively [17–24] (Table 2). The p*I* of the purified enzyme was measured by 2-DE to be around 4.4, which is different from the p*I* (5.25) of the ginsenoside β-glucosidase from *Fusobacterium* K-60 [18].

Table 1
Purification of a β-glucosidase (FPG) from *F. proliferatum* ECU2042.

Step	Total activity[a] (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification factor
Crude extract	989	203	4.86	100	1
$(NH_4)_2SO_4$ precipitation	1979	77.1	25.7	200	5.3
Butyl-Toyopearl	943	23.3	40.4	95.4	8.3
DEAE-Toyopearl	797	10.3	77.5	80.6	16.0
TSK-gel	404	1.18	344	40.9	70.7
SDS-R-PAGE (1)	21.6	0.027	803	2.18	165
SDS-R-PAGE (2)	17.2	0.007	2458	1.74	506

[a] Activity was measured with ginsenoside Rg_3 as substrate.

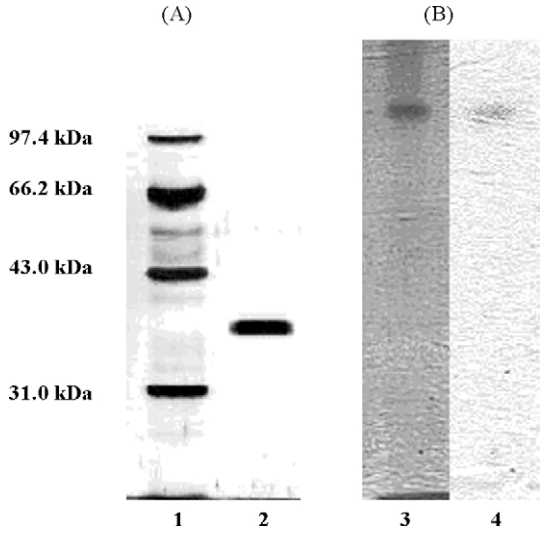

Fig. 1. Electrophoresis of a β-glucosidase (FPG) purified from *Fusarium proliferatum* ECU2042. (A) SDS-PAGE with silver staining: lane 1, protein markers: rabbit phosphorylase b 97.4 kDa, bovine serum albumin 66.2 kDa, rabbit actin 43.0 kDa and bovine carbonic anhydrase 31.0 kDa; lane 2, purified enzyme. (B) SDS-R-PAGE: lane 3, silver staining; lane 4, activity staining.

3.2. Biochemical characterization of FPG

3.2.1. Effects of pH and temperature

The purified enzyme exhibited the maximum activities at pH 5.0 (Fig. 2A) and 50 °C (Fig. 2B), which were similar with the ones from FFCDL-48 [17], the China white jade snail [23] and *Aspergillus* sp. g48p [24], as listed in Table 2. Though their optimal pHs were similar with this FPG, the optimal temperatures of the ginsenoside β-glucosidases from ginseng [19], *Aspergillus* sp. 848g [20] were 60 and 40 °C, respectively. Differently, the ginsenoside β-glucosidase from *Fusobacterium* K-60 had the optimal activity at pH 6.0 and 40 °C [18], while the one from China white jade snail occurred at pH 5.6 and 70 °C [22].

Within the pH range of 3.0–7.0, our purified enzyme was stable at pH 4.0–6.5, and the enzyme activity remained more than 70% after incubating in these pHs at 4 °C for 24 h (data not shown). The pH stability was similar with the ginsenoside β-glucosidase from *Aspergillus* sp. g48p [24], but a little poorer than the ones from China white jade snail [22,23] (Table 2).

The plot of natural logarithm of the residual activity ($\ln R$) versus the time of preincubation at the indicated temperatures showed that the thermal inactivation of FPG follows first-order kinetics, and the inactivation rate coefficient (k_d) equals to the absolute value of the slope in the regression line (Fig. 3). Activation energy for denaturation (E_d) of FPG was determined from plotting of $\ln k_d$ against $1/T$ by fitting the data via linear regression to an Arrhenius-type equation [25], and was calculated to be 88.6 kJ mol^{-1}. The half-lives of the purified enzyme at 30, 40, 50, 60 and 70 °C were 141.4, 90.0, 58.7, 27.1 and 1.4 h, respectively. To the best of our knowledge, the thermal stability of this enzyme (FPG) was better than most of those isolated from other mesophilic microorganisms, except for the ones from thermophiles.

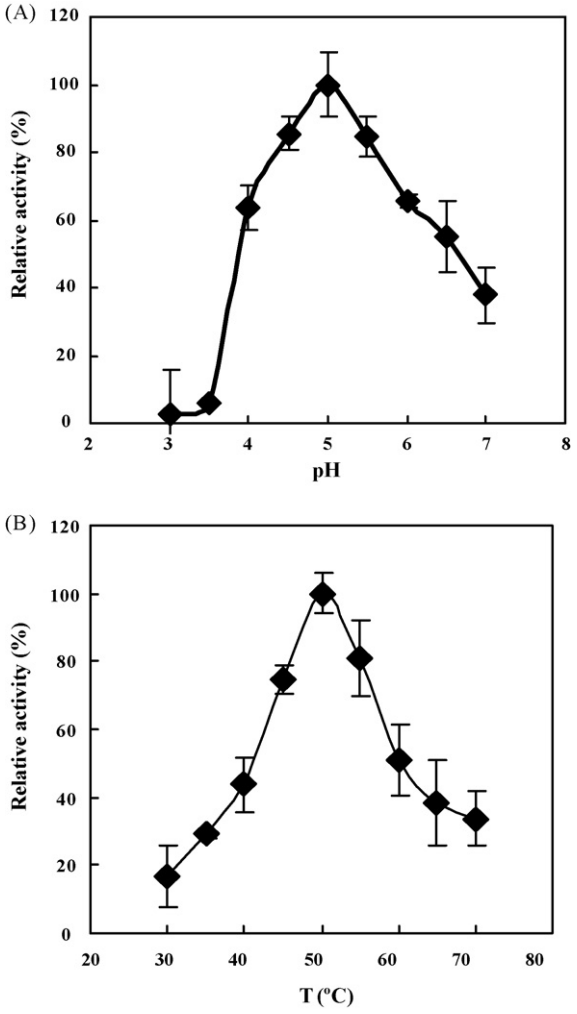

Fig. 2. Effect of pH (A) and temperature (B) on activity of *Fusarium proliferatum* β-glucosidase (FPG): (A) the enzyme activity measured at pH 5.0 was taken as 100%; (B) the enzyme activity measured at 50 °C was taken as 100%. The enzyme activities were measured as described in Section 2.7. All experiments were performed in duplicate.

3.2.2. Effect of metal ion and EDTA

The influence of various metal ions and EDTA (at 10 mM) on the purified enzyme activity was shown in Table 3. Except for Co^{2+} and

Table 2
Comparison between *F. proliferatum* β-glucosidase (FPG) and other ginsenoside β-glucosidases reported.

Source	Molecular mass (kDa)	p*I*	Optimal pH	pH stability	Optimal temp. (°C)	Temp. stability (°C)	Reference
FFCDL-48	34 (monomer)	N.A.[a]	5.0	N.A.	50	N.A.	[17]
Fusobacterium K-60	320 (tetramer)	5.25	6.0	N.A.	40	N.A.	[18]
Ginseng	59 (monomer)	N.A.	5.0	N.A.	60	<60	[19]
Aspergillus sp. 848g	31 (monomer)	N.A.	5.0	3.0–7.0	40	30–50	[20]
Helix snailase	460 (tetramer)	N.A.	N.A.	N.A.	N.A.	N.A.	[21]
China white jade snail	230 (dimer)	N.A.	5.6	3.0–11.0	70	30–70	[22]
China white jade snail	220 (dimer)	N.A.	5.0	4.0–10.0	50	30–50	[23]
Aspergillus sp. g48p	80 (monomer)	N.A.	5.0	4.5–7.0	50	20–40	[24]
Fusarium sp. ECU2042	78.7 (dimer)	4.4	5.0	4.0–6.5	50	<60	This work

[a] N.A: not available.

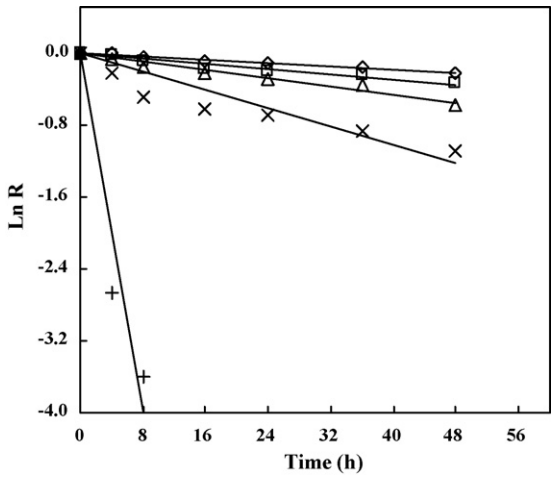

Fig. 3. First-order plots of thermal denaturation effect on *F. proliferatum* β-glucosidase (FPG). Samples were incubated at 30 °C (◊), 40 °C (□), 50 °C (△), 60 °C (×) and 70 °C (+) in 0.2 ml of NaAc/HAc buffer, pH 5.0, and the aliquots withdrawn at different time intervals were cooled on ice before assay of residual activity at 50 °C. All results are the means of two independent experiments.

Zn^{2+}, Ni^{+}, Mg^{2+}, Ca^{2+} and Mn^{2+} all had a little negative effect on FPG. Different from general enzymes, FPG was not apparently affected by heavy metal salts, such as Cu^{2+} and Fe^{3+}, but Fe^{2+} significantly inhibited FPG.

The chelating agent EDTA also reduced FPG activity, suggesting that the purified enzyme may depend on or be sensitive to some metal ions.

3.2.3. Substrate specificity and biohydrolysis of ginsenosides

At first, initial rates of the purified enzyme hydrolyzing ginsenoside Rg_3, cellobiose, pNPG and *p*-nitrophenyl β-D-galactopyranoside were examined. The activities on cellobiose and *p*-nitrophenyl β-D-galactopyranoside were too low to be detected, and the initial rate of hydrolyzing ginsenoside Rg_3 was about 15 times that of hydrolyzing pNPG (158 nmol h^{-1} mg $protein^{-1}$). The data indicates that FPG is not only specific for β-glucoside, but also especially fond of some aglycones. When using different alkyl glucosides as substrates (Table 4), the hydrolytic ability increased with the extension of alkyl chain except isopropyl β-D-glucopyranoside. This result suggests that the active center of the enzyme should be relatively hydrophobic. Additionally, we also used various natural glycosides, as well as different aryl glucosides as substrates to study the substrate specificity (Table 4). Various levels of activities on these substrates show that the purified enzyme has a wide spectrum of specificity on various types of β-glucosides.

Table 3
Effect of metal ion and EDTA on activity of *F. proliferatum* β-glucosidase (FPG)[a].

Metal ions (10 mM)	Relative activity[b] (%)
None	100 ± 4
Ni^{+}	74 ± 15
Mg^{2+}	75 ± 0
Ca^{2+}	73 ± 8
Mn^{2+}	77 ± 6
Fe^{2+}	34 ± 21
Co^{2+}	114 ± 9
Cu^{2+}	96 ± 8
Zn^{2+}	92 ± 6
Fe^{3+}	107 ± 0
EDTA	67 ± 5

[a] The enzyme reactions were carried out in the standard reaction mixture consisting of 100 μl of enzyme solution (the protein concentration was 0.039 mg ml^{-1}), 80 μl of NaAc/HAc buffer (0.5 M, pH 5.0) and 20 μl of ginsenoside Rg_3 solution (5 mg ml^{-1}, in water), with 10 mM metal ion or EDTA.

[b] The enzyme activity measured without metal ion or EDTA was taken as 100%.

Table 4
Relative activities of *F. proliferatum* β-glucosidase (FPG) on different substrates[a].

Substrate	Concentration	Relative activity (%)[b]
Ginsenside Rg_3	5 mg ml^{-1}	100 ± 7
Ethyl β-D-glucopyranoside	10 mM	14.6 ± 0.0
Propyl β-D-glucopyranoside	10 mM	31.7 ± 2.4
Isopropyl β-D-glucopyranoside	10 mM	9.8 ± 0.0
Propenyl β-D-glucopyranoside	10 mM	26.8 ± 2.4
Butyl β-D-glucopyranoside	10 mM	39.0 ± 0.0
Hexyl β-D-glucopyranoside	10 mM	53.7 ± 4.9
Cyclopentyl β-D-glucopyranoside	10 mM	65.9 ± 2.4
4-Bromophenyl β-D-glucopyranoside	10 mM	n.d.[c]
4-Cholrphenyl β-D-glucopyranoside	10 mM	227 ± 2
4-Hydroxybenzyl β-D-glucopyranoside	10 mM	551 ± 34
4-Methoxybenzyl β-D-glucopyranoside	10 mM	73.2 ± 4.9
Salidroside	10 mM	51.2 ± 2.4
Esculin hydrate	5 mg ml^{-1}	31.7 ± 7.3
Barboloin	5 mg ml^{-1}	63 ± 15
Soy saponins	10 mg ml^{-1}	117 ± 5
Ginsenoside Rh_2	5 mg ml^{-1}	n.d.

[a] Enzyme activities were determined by the amount of glucose released from these glucosides. Results are expressed as the means of two independent measurements. Controls were done similarly by omitting the substrate or enzyme.

[b] The enzyme activity (19.1 μmol h^{-1}) on ginsenoside Rg_3 was defined as 100%, and the protein concentrations of the enzyme solution used to analyze the enzyme activity was 0.039 mg ml^{-1}.

[c] Not detected.

To verify ginsenoside Rg_3 biotransformation by FPG, ginsenoside Rg_3 was incubated with FPG. The hydrolyzed product was periodically assayed by HPLC. As shown in Fig. 4, when Rg_3 was used as the substrate, only one product was detected and identified as ginsenoside Rh_2 (as described previously [11]). The amount of Rh_2 increased with the extension of the reaction time, while another hydrolysis product, protopanaxadiol (PPD), was not detected after incubation at 50 °C for 24 h. Subsequently, we also used ginsenoside Rh_2 as a substrate to verify the specificity of FPG. As expected, the enzyme did not hydrolyze Rh_2 after incubation at 50 °C for 24 h (Table 4), suggesting that this enzyme acts specifically on the glycosidic bond between the two glucose residues of the ginsenoside Rg_3, just like the ones from FFCDL-48 [17], ginseng [19] and *Aspergillus* sp. 48 g [20]. In contrast, the other two glucosidases, the ginsenoside β-glucosidases from FFCDL-00 [17] and *Aspergillus* sp.

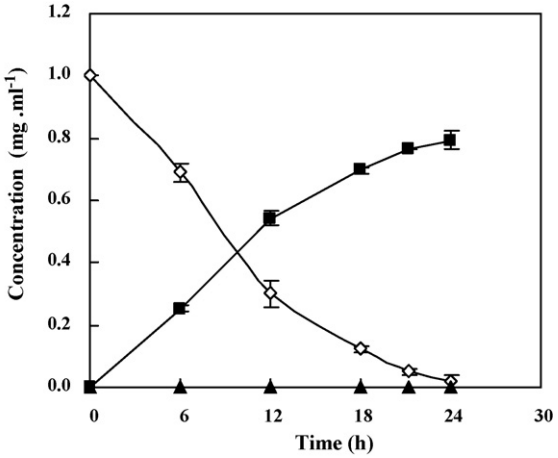

Fig. 4. Time course of ginsenosides Rg_3 hydrolyzed by *F. proliferatum* β-glucosidase (FPG). The HPLC and reaction conditions were described in Section 2. Open diamonds, ginsenoside Rg_3; filled squares, ginsenoside Rh_2; filled triangles, protopanaxadiol (PPD).

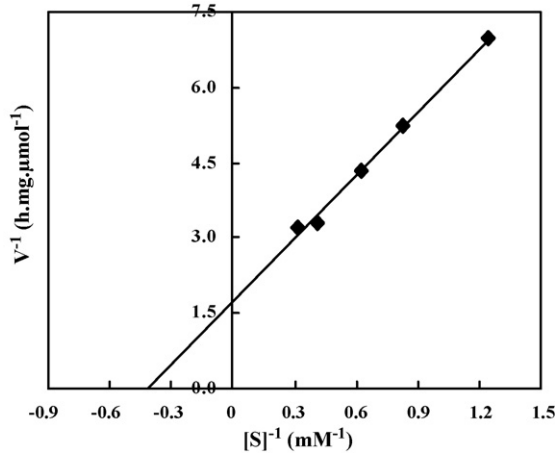

Fig. 5. Lineweaver–Burk plot of FPG. All results are the means of two independent experiments.

848 g [20] could non-selectively hydrolyze Rg_3 and Rh_2, yielding a mixture of Rh_2 and protopanaxadiol.

Using a Lineweaver–Burk plot (Fig. 5), the apparent K_M and V_{max} values of the purified FPG towards ginsenoside Rg_3 were calculated to be 2.37 mM and 0.568 μmol (h mg protein)$^{-1}$, respectively, at pH 5.0 and 50 °C.

4. Conclusions

In this study, through six steps of purification process, we have successfully got a pure β-glucosidase (FPG) from *F. proliferatum* ECU2042 (or CGMCC 1495), which was a homogeneous dimer of 78.7 kDa with a p*I* of 4.4. The purified enzyme exhibited optimal activities at pH 5.0 and 50 °C, and high degrees of resistance against unfavorable conditions such as pH, temperature and some heavy metal ions. Except for transforming ginsenoside Rg_3 into Rh_2, the purified enzyme exhibits a relatively wide but unique specificity to other substrates. The properties described here differ from those reported hitherto in at least one of the following aspects: molecular mass, p*I*, optimal temperature pH, and substrate specificity. Therefore, the purified enzyme is considered to be a novel and unique ginsenoside β-glucosidase.

The β-glucosidase (FPG) obtained from *F. proliferatum* ECU2042 is considered to be a promising biocatalyst for practical application. Its high activity, broad substrate specificity and good stability make this enzyme to be a good candidate for use in biotransformation for different purposes, such as new drug discovery and development of natural medicines.

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