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Original Article

Purification and characterization of a novel and unique ginsenoside Rg_1-hydrolyzing β-D-Glucosidase from *Penicillium sclerotiorum*

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In this paper, a novel and unique ginsenoside Rg_1-hydrolyzing β-D-Glucosidase from *Penicillium sclerotiorum* was isolated, characterized, and generally described. The β-Glucosidase is an ∼180 kDa glycoprotein with pI 6.5, and consists of four identical subunits of ∼40 kDa. The β-Glucosidase was active in a narrow pH range (4–5) and at relatively high temperature (60–70°C). The optimal activity against *p*-nitrophenyl-β-D-glucopyranoside (pNPG) was as follows: pH 4.5 and temperature 65°C. Under these conditions, the K_m of the enzyme was 0.715 mM with a V_{max} of 0.243 mmol nitrophenol/min mg. Metal ions such as Ba^{2+}, K^+, Fe^{3+}, and Co^{2+} significantly promoted the enzymatic activity, while Ca^{2+}, Mg^{2+}, and Ag^+ inhibited its activity. Of the tested substrates, only ginsenoside Rg_1 could be specifically hydrolyzed by the β-Glucosidase at the C6-glucoside to form the rare ginsenoside F_1. These properties were novel and different from those of other previously described glycosidases.

Keywords β-D-Glucosidase; *Penicillium sclerotiorum*; ginsenoside; enzyme property

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Introduction

Ginseng (root of *Panax ginseng* C.A. Meyer, Family *Araliaceae*) has been used as a traditional medicine for preventive and therapeutic purposes in Asian countries for >2000 years. One of the physiologically active materials of ginseng plants is saponin (ginsenoside), and to date >40 types of ginsenosides are known [1]. There are three types of ginsenosides: protopanaxadiol (PPD)-type, protopanaxatriol (PPT)-type, and oleanonic acid-type saponins such as the ginsenoside Ro. The ginsenosides Rb_1, Rb_2, Rb_3, Rc, Rd, F_2, Rg_3, and Rh_2 are PPD-type ginsenosides; Re, Rg_1, Rg_2, Rg_4, Rh_1, and Rh_4 are PPT-type ginsenosides; these ginsenosides are dammarane-type saponins. The main ginsenosides in the ginseng are the ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf, and Rg_1; others, such as Rg_3, Rg_2, Rg_5, Rh_1, Rh_3, Rh_4, and F_1, are minor ginsenosides. The minor ginsenosides have special physiological activities. For example, the ginsenosides Rh_2, Rh_3, Rg_3, and Rh_1 have good anticancer properties with very low side effects, while the ginsenosides Rg_3 and Rg_2 have anti-thrombus and endothelium-mediating activity [2]. Thus, the minor ginsenosides are very useful as drugs and healthy foods. However, it is very difficult to obtain the minor ginsenosides from red and wild ginseng, because their contents are very low.

β-Glucosidase (EC 3.2.1.21) constitutes a major group among the glucoside hydrolases [3]. The glucoside hydrolases occur ubiquitously in all three (archea, eubacteria, and eukarya) domains of living organisms and have been the focus of many recent researches, because of their important roles in a variety of fundamental biological processes [4]. The β-Glucosidases show similar specificity for their β-glucoside substrates, but distinct specificities for the aglycone linked to the glucosyl group, suggesting their diverse biological functions [5].

In our preliminary study, we reported the microbial transformation of the ginsenosides Rb_1, Rb_3, and Rc to compound K (C-K), compound Mx (C-Mx), and the ginsenoside Mc (G-Mc) by *Fusarium sacchari* [6]. Later, we screened another filamentous fungus, *Penicillium sclerotiorum*, which can specifically transform the ginsenoside Rg_1 (GRg_1) to the rare ginsenoside F_1 (GF_1) [7]. GF_1 was reported to induce the reduction of gap junction intercellular communication [8], and it showed stronger inhibition activity than ginsenoside Rh_1 with respect to the proliferation of human osteosarcoma cells U2OS [9]. Additionally, GF_1 could significantly reduce ultraviolet UV-B-induced cell death and protect HaCaT cells from apoptosis [10]. However, it was very difficult to obtain GF_1 from red and wild ginseng due to its very low content. Therefore, we purified and characterized the novel specific β-Glucosidase produced by *P. sclerotiorum* as a new way of preparing GF_1.

Materials and Methods

Strains, media, and materials

Penicillium sclerotiorum was originally separated from soil and preserved in our laboratory. The preparation of slant medium, seed medium, and transformation medium was described previously [7].

The ginsenosides were purchased from Saixun Biotech Co., Ltd. (Kunming, China). *p*-Nitrophenyl-β-D-glucopyranoside (pNPG), 4-methylumbelliferyl-β-D-glucopyranoside (MUG), periodic acid-Schiff (PAS), acrylamide, sodium dodecyl sulfate (SDS), standard proteins including thyroglobulin (669 kDa), myosin (440 kDa), catalase (250 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), and cytochrome c (12 kDa) were all obtained from Sigma (St. Louis, USA). Methylene-bis-acrylamide (Bis) and tetramethylethylenediamine were obtained from Fluka Co., Ltd. (Buchs, Switzerland). The protein marker was obtained from Watson Biotechnology Co., Ltd (Beijing, China). Bromophenol blue was purchased from BBI Co., Ltd. (Gaithersburg, USA). Coomassie brilliant blue R-250, DEAE Sepharose fast flow, SP Sepharose fast flow, Q Sepharose fast flow, and Sephacryl S-300 were ordered from GE Healthcare Co., Ltd. (Waukesha, USA). Other general chemicals used were of analytical reagent grade.

Preparation of crude enzyme

Eight milliliters of spore suspension (5×10^7 spores/ml) of *P. sclerotiorum* were transferred into the transformation medium (80 ml) per 500-ml flask, and a total of 12 flasks were cultured together in a shaker for 90 h at 28°C. The mycelial supernatant was collected by centrifugation and concentrated by ultrafiltration (the material of the membrane was polyethersulfone (PES) and the molecular weight cut-off of the membrane was 100–500 kDa). Then $(NH_4)_2SO_4$ powder was added up to 20% saturation to the concentrated supernatant to precipitate some proteins that were removed by centrifugation later. $(NH_4)_2SO_4$ powder was continually added to 80% saturation and the mixture was kept at 4°C overnight. Then, the mixture was centrifuged to collect protein precipitates, and the precipitates were dialyzed against HAc–NaAc buffer (0.02 M, pH 4.8). Finally, the crude enzyme was obtained by lyophilizing the dialysate.

Purification of β-D-Glucosidase

The crude enzyme was first applied to a DEAE Sepharose fast flow column (Φ2.6 cm × 10 cm) (column 1), which had been pre-equilibrated with 0.05 M phosphate-buffered saline (PBS) (pH 8.0). The column was eluted with a linear gradient of 0–0.2 M NaCl in 0.05 M PBS (pH 8.0) for ~5 column volumes at a flow rate of 1.3 ml/min. The fractions from column 1 were collected and assayed for activity, and only the active fraction was sequentially loaded onto an SP Sepharose fast flow column (Φ1.6 cm × 20 cm; column 2), which had been pre-equilibrated with 0.02 M HAc–NaAc (pH 4.8) buffer. The column was eluted by a linear gradient of 0–0.2 M NaCl in 0.02 M HAc–NaAc (pH 4.8) for ~10 column volumes at the rate of 1.5 ml/min. Finally, the active component from column 2 was further purified by a Q Sepharose fast flow column (Φ1.6 cm × 20 cm; column 3). It was eluted by a linear gradient of 0–0.2 M NaCl in 0.05 M Tris–HCl (pH 8.0) with a flow rate of 1.5 ml/min. The active component from column 3 was fully dialyzed and lyophilized to obtain the purified β-Glucosidase [11–13].

Activity assay of β-D-Glucosidase against pNPG, GRg_1, and MUG

About 20 μl of β-D-Glucosidase solutions were added to a mixture of 260 μl citric acid buffer (0.1 M; pH 4.5) and 20 μl pNPG (4×10^{-3} M). The mixture was incubated at 65°C for 30 min, and 1.5 ml NaOH (0.25 M) was added. Finally, the optical density (OD) was measured at 405 nm with a UV–visible spectrophotometer (JASCO V-530, Tokyo, Japan). One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per minute under the above conditions.

High-performance liquid chromatography (HPLC; Shimadzu, Tokyo, Japan) was used for monitoring the hydrolyzing activity of the β-D-Glucosidase to transform GRg_1 to GF_1. The HPLC chromatograph was performed as follows: the column temperature was 40°C, the mobile phase was methanol/water (68/32; *v*/*v*), the flow rate was 1 ml/min, the injection volume was 20 μl, and the detection wavelength was selected at 203 nm [14]. The data were processed using the processing software (Zhejiang University N2000, Hangzhou, China).

Activity staining of β-D-Glucosidase in a slab gel was performed with 5% of MUG on an 8% native polyacrylamide gel electrophoresis (PAGE) gel [15]. Before detection of enzymatic activity, the gels were washed three times with 50 mM citrate acid–sodium dihydrogen phosphate (pH 4.5) and incubated at 50°C for 20 min in the same solution containing 5% MUG. The β-D-Glucosidase activity was assayed under UV light at 254 nm [11].

Determination of molecular weight and pI of Glucosidase

The molecular mass of β-Glucosidase was estimated by both SDS–PAGE [15] and gel filtration on a column of Sephacryl S-300 HR (Φ1.0 cm × 40 cm).

SDS–PAGE was performed on an 8% polyacrylamide gel in a Mini-Protean III dual-slab cell electrophoresis unit

(Bio-Rad, Hercules, USA), and the proteins were visualized with Coomassie brilliant blue R250.

The gel filtration column was equilibrated with 20 mM Tris–HCl buffer (pH 7.0) and calibrated by the elution of standard protein markers, which included thyroglobulin (669 kDa), myosin (440 kDa), catalase (250 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), and cytochrome c (12 kDa).

The pI of the β-Glucosidase was determined by the isoelectric focusing (IEF) method [16].

The sugar moieties of the purified enzyme were detected on SDS–PAGE by PAS staining [17].

Effects of pH, temperature, and metal ions on enzymatic activity

Enzymatic activity was investigated at a pH range of 2.5–6.0 in 0.1 M citric acid buffer against pNPG. The temperature effect was determined between 30 and 80°C at the optimal pH. Different metal ions (Na^+, K^+, Li^+, Mg^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Al^{3+}, Ca^{2+}, Zn^{2+}, Ba^{2+}, and Ag^+) were separately added to the enzyme reaction system at a concentration of 1 mM at the optimal pH and temperature. The enzyme reaction system was incubated for 2 h to determine its residual activity. The maximal enzymatic activity was observed at pH 4.5 and 65°C respectively, when incubated for 2 h and was set as 100%. Values were expressed as the mean ± SD ($n = 3$).

Kinetic studies

The activity of β-Glucosidase was measured against pNPG under optimal conditions, with the final concentration from 0.1 to 3.2 mM. Measurements were carried out in triplicate. The K_m and V_{max} values were calculated by a Lineweaver–Burk plot [18].

Substrate specificity of β-D-Glucosidase

Some ginsenosides with a C6-glucoside, such as GRg_2, GRg_1, and p-F11, were hydrolyzed separately as substrates by the purified β-Glucosidase. The products were detected by thin-layer chromatography (TLC) and HPLC. The TLC chromatograph was developed with a chloroform/methanol/water (7:3:1; *v*/*v*/*v*) solution and then colored by a 10% sulfuric acid–ethanol solution. The HPLC chromatograph was performed as described above.

Results

Purification of the enzyme

A total of 1.22 g crude enzyme was successfully prepared from 960 ml of culture broth of *P. sclerotiorum*. Three components were obtained when 1.0 g crude enzyme was first executed on DEAE-Sepharose column 1. Only component I-1 appeared at the elution gradient of 0.021 M NaCl, showing hydrolyzing activity on pNPG and GRg_1. Thus, component I-1 was collected, condensed, and processed to SP-Sepharose column 2. Two components were obtained from SP-Sepharose column 2, and component II-2, corresponding to an elution gradient of 0.069 M NaCl, was the active fraction when tested on pNPG and GRg_1. So, component II-2 was further applied to Q-Sepharose column 3. Component III-1 was confirmed as the active fraction in the elution gradient of 0.087 M NaCl. After these three steps, the enzyme was purified 94.69-fold with the specific activity of 902.46 U/mg protein, and the yield was 35.23% (**Table 1**). Moreover, the enzymatic activity detected by MUG *in situ* on a non-denaturing PAGE gel revealed a positive fluorescent band under UV light, which further confirmed that the purified enzyme had β-D-Glucosidase activity (**Fig. 1**, line 5).

Molecular weight, pI, and composition of β-D-Glucosidase

The purified enzyme was analyzed by denaturing SDS–PAGE. Results showed only one electrophoretic band corresponding to ~40 kDa (**Fig. 1**, line 2). This confirmed that the purified enzyme had reached homogeneity and electrophoretic purity. However, when the native purified enzyme was applied on a non-denaturing PAGE, there was still one protein band. But its molecular weight exceeded the range of protein marker (>116 kDa) (**Fig. 1**, line 4). Therefore, the molecular weight of the native β-D-Glucosidase was further investigated by Sephacryl S-300 HR gel filtration chromatography. The elution volumes (*Vt*/*Vo*) of different standard proteins [thyroglobulin (669 kDa), myosin

Table 1 Purification of β-D-Glucosidase from *P. sclerotiorum*

Purification steps	Total protein (mg)	Total activity (U) against pNPG	Specific activity (U/mg protein) against pNPG	Purification fold	Yield (%)
Crude enzyme	1000	9530.5	9.53	1.00	100.00
DEAE Sepharose fast flow	35.28	8149.5	230.99	24.23	85.51
SP Sepharose fast flow	9.18	4467.9	486.69	51.06	46.88
Q Sepharose fast flow	3.73	3366.2	902.46	94.69	35.23

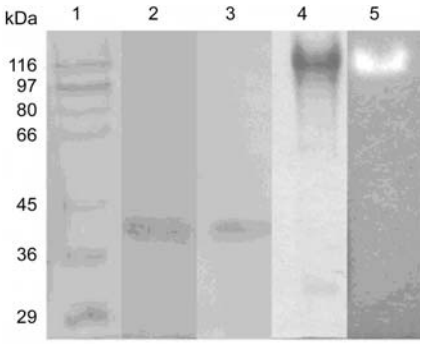

Figure 1 Electrophoresis of the purified enzyme Lane 1, molecular weight marker; lane 2, purified enzyme electrophoresed by SDS–PAGE; lane 3, purified enzyme electrophoresed by SDS–PAGE with PAS straining; lane 4, purified enzyme electrophoresed by non-denatured PAGE with Coomassie brilliant blue straining; lane 5, purified enzyme electrophoresed by non-denatured PAGE with MUG staining.

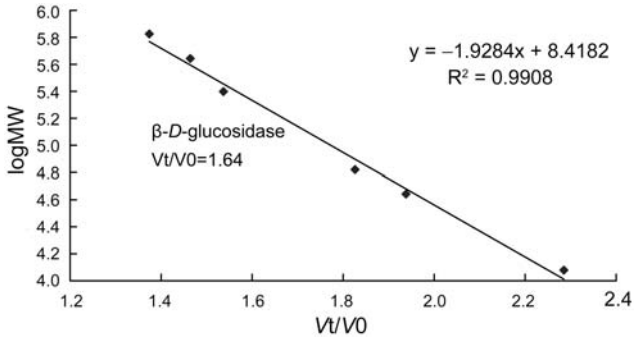

Figure 2 Standard curve of molecular weight It was used for calculating the molecular weight as determined by Sephacryl S-300 HR gel filtration chromatography.

(440 kDa), catalase (250 kDa), BSA (66 kDa), ovalbumin (44 kDa), and cytochrome c (12 kDa)] were first measured. Then the standard curve of molecular weight was drawn as shown in **Fig. 2**. Under the same conditions, the *Vt*/*V*0 value of the native enzyme was measured as 1.64, and its molecular weight was calculated as ~180 kDa. So, the molecular weight determined by denaturing SDS–PAGE (~40 kDa) was significantly different from gel filtration chromatography (~180 kDa). From these results, we presumed that the native enzyme was an oligomeric protein of 180 kDa with four identical subunits of ~40 kDa each.

Moreover, the purified enzyme was tested as to whether there was sugar modification in the protein molecule by PAS staining on PAGE. The red–purple band of the purified enzyme showed positive PAS reaction, which indicated that it was a glycoprotein (**Fig. 1**, line 3).

The standard pI curve in the IEF gel in the range of pH 3.5–10 was drawn and is depicted in **Fig. 3**. The pI value of the β-Glucosidase was calculated according to its migration distance (3.62 cm) and was calculated to be 6.5.

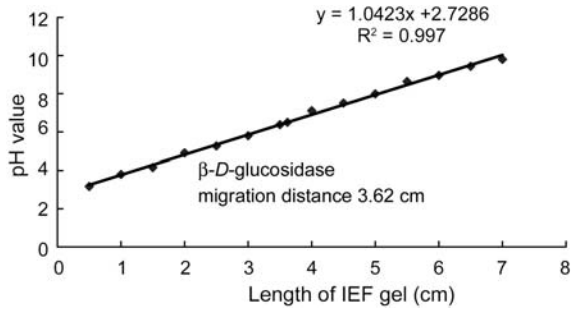

Figure 3 Standard curve of pI in IEF gel The pI value of β-Glucosidase was calculated to be 6.5.

Effects of pH, temperature, and metal ions on enzymatic activity

Results showed that the optimum pH for β-Glucosidase was 4.5, and the optimum temperature was 65°C (**Fig. 4**). The enzyme retained relatively high activity at the narrow pH range of 4–5 and at temperatures of 60–70°C, which was significantly different from some known β-Glucosidases [11,14].

Metal ions greatly affect the enzyme activity. At a concentration of 1 mM, Ba^{2+}, K^{+}, Fe^{3+}, and Co^{2+} significantly improved the enzyme activity by 51%, 31%, 29% and 22%, respectively, while Ca^{2+}, Mg^{2+}, and Ag^{+} inhibited the enzyme activity by 89%, 73%, and 48% at the same concentration (**Fig. 5**).

Kinetic parameters

At the optimal pH of 4.5 and temperature of 65°C, the K_m and V_{max} of enzyme activity against pNPG were calculated as 0.715 mM and 0.243 mmol nitrophenol/min/mg, respectively (**Fig. 6**). The K_m value was different from that of other β-Glucosidases, such as G II (0.224 mM) [14] and G IV (0.338 mM) [19], when tested against pNPG.

Substrate specificity of β-Glucosidase

Some of the ginsenoside compounds, such as PPD-type ginsenosides Rb_1, F2, Rh_2 and PPT-type ginsenosides Rg_1, Rg_2, Rh_1, Rg_1, Rf, as well as ginsenosides p-F11 were used to study the substrate specificity of β-Glucosidase under optimal pH and temperature conditions. The hydrolyzed products were detected by TLC. Only ginsenoside Rg_1 was hydrolyzed and its biotransformation product appeared as a new spot in the TLC plate; the other ginsenosides, along with the C3-, C6-, and C 20 glycosides, could not be hydrolyzed. The hydrolyzed product of GRg_1 was further detected by HPLC, and only one type of product appeared (**Fig. 7**); the latter was further identified as rare ginsenoside F_1 [7]. These results demonstrated that the β-D-Glucosidase from *P. sclerotiorum* had substrate specificity and was distinguishable from the three-dimensional structure of ginsenosides. It could only

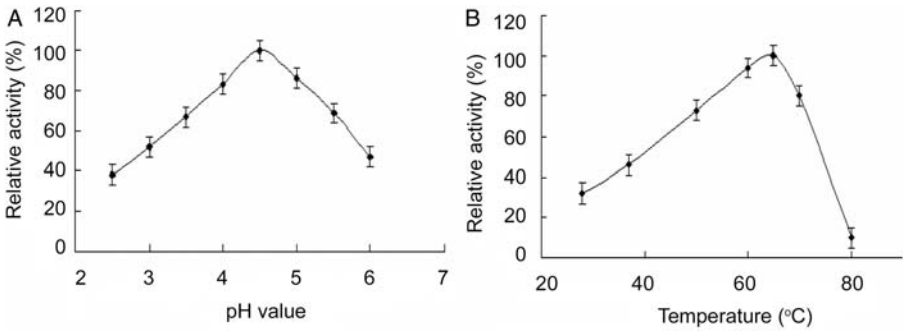

Figure 4 Effects of pH and temperature on the activity of β-Glucosidase (A) pH and (B) temperature. The maximum activity was observed at pH 4.5 and 65°C respectively, and was set as 100%. The values were expressed as mean ± SD ($n = 3$).

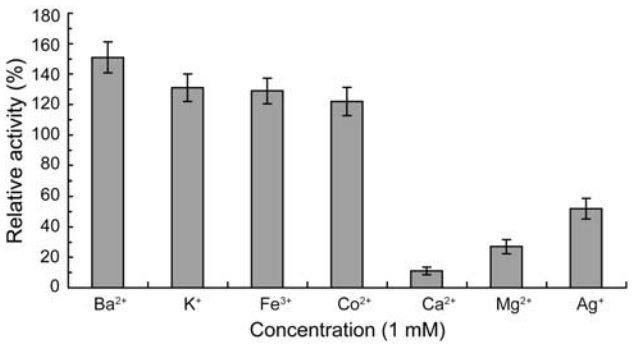

Figure 5 Effects of metal ions on enzymatic activity The concentration of different metal ions is 1 mM. They have different effects on enzyme activity.

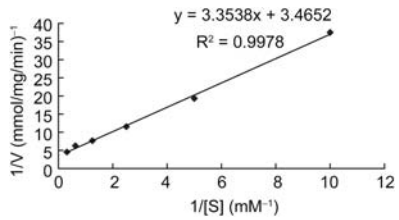

Figure 6 Lineweaver–Burk plots for the hydrolysis of pNPG by purified enzyme K_m and V_{max} of the enzyme activity against pNPG were calculated as 0.715 mM and 0.243 mmol nitrophenol/min/mg, respectively.

hydrolyze glycoside at the C-6 position of ginsenosides GRg_1, but could not hydrolyze the C-20 glycoside of the ginsenosides.

Discussion

The ginsenoside F1 was discovered early in 1976, but its pharmacology activity was only reported several years ago [8–10] because it is only present in wild ginseng at relatively low concentrations [20], or appears in the intestine of human and animals by the *in vivo* hydrolysis of enteric bacteria, such as cteroides JY6, Fusobacterium K-60,

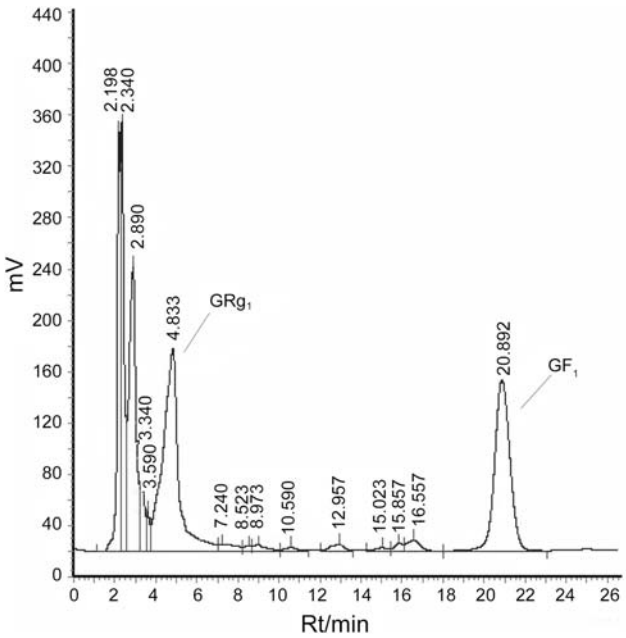

Figure 7 HPLC analysis of ginsenosides GRg_1 and its enzymatic product ginsenosides GF1 It showed that only one type of product appeared.

Bifidobacterium K-103, Bifidobacterium K-525, and Bacteroides HJ15 [21].

Different types of ginsenoside-hydrolyzing β-Glucosidases have been reported in recent years. For example, Yu *et al*. discovered a ginsenosidase of ~80 kDa capable of hydrolyzing multi-glycosides of ginsenoside from *Aspergillus* sp. g48p that was named ginsenoside type I [22]. It can hydrolyze different glycosides of protopanaxadiol-type ginsenosides, i.e. 3(carbon)-*O*-β-glucoside of Rb_1, Rb_2, Rb_3, Rc, Rd, or the 20(carbon)-*O*-β-glucoside of Rb_1, 20(carbon)-*O*-β-xyloside of Rb_3, 20(carbon)-*O*-α-arabinoside of Rb2, and 20(carbon)-*O*-α-arabinoside of Rc to mainly produce F_2, a compound-K (C-K), and Rh_2. However, the ginsenosidase cannot hydrolyze the C-6 glycosides of the PPT-type ginsenosides, such as Re, Rf, Rg_1, and Rg_2 [22].

Hu *et al*. reported another type of β-Glucosidase with broad regiospecific activity that was designated G II; it was purified using acetone from the viscera of the Chinese white jade snail (*Achatina fulic*a) [11]. G II consists of two identical subunits (110 kDa) with a native molecular mass of 220 kDa. It can cleave both the β-(1→2)-glucosidic linkage at 3-C and the β-(1→6)-glucosidic linkage at 20-C of ginsenosides to convert the ginsenosides Rb_1, Rb_2, Rb_3, and Rc into their active metabolites, compound-K, compound-Y, Mx, and Mc, respectively [11,23].

Interestingly, *Penicillium* sp. were known to secrete some glycosidases, such as naringinase from *P. decumben*s, hydrolyzing the ginsenosides Re and Rg_1 to produce ginsenoside F_1 and other catabolites, like the ginsenosides Rf, Rg_2, Rh_1, which showed non-specific hydrolysis activity [24]. Recently, a xylanase from *P. sclerotiorum* was also investigated [25].

This investigation is the first one to report the purification of a novel oligomeric β-Glucosidase from *P. sclerotiorum* capable of specifically hydrolyzing the PPT-type ginsenoside Rg_1. This β-Glucosidase can hydrolyze the C-6 glycoside of the ginsenoside Rg_1 to form the rare ginsenoside F_1, but can not hydrolyze the C-20 glycoside of the ginsenoside Rg_1 to produce Rh_1. Moreover, it also cannot hydrolyze other compounds with similar chemical structures. In other words, β-Glucosidase can be conveniently used to prepare the rare ginsenoside F_1 from the panaxatriol-type ginsenoside Rg_1 on a large scale *in vitro* or by *P. sclerotiorum*.

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