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Purification and characterization of a highly selective glycyrrhizin-hydrolyzing β-glucuronidase from *Penicillium purpurogenum* Li-3

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ABSTRACT

A novel β-glucuronidase from filamentous fungus *Penicillium purpurogenum* Li-3 was purified to electrophoretic homogeneity by ultrafiltration, ammonium sulfate precipitation, DEAE-cellulose ion exchange chromatography, and Sephadex G-100 gel filtration with an 80.7-fold increase in specific activity. The purified β-glucuronidase is a dimeric protein with an apparent molecular mass of 69.72 kDa (m/z = 69,717), determined by MALDI/TOF-MS. The optimal temperature and pH of the purified enzyme are 40 °C and 6.0, respectively. The enzyme is stable within pH 5.0–8.0, and the temperature up to 45 °C. Mg^{2+} ions enhanced the activity of the enzyme, Ca^{2+} and Al^{3+} showed no effect, while Mn^{2+}, Zn^{2+}, Hg^{2+} and Cu^{2+} substantially inhibited the enzymatic activity. The K_m and V_{max} values of the purified enzyme for glycyrrhizin (GL) were evaluated as 0.33 mM and 59.0 mmol mg^{-1} min^{-1}, respectively. The purified enzyme displayed a highly selective glycyrrhizin-hydrolyzing property and converted GL directly to glycyrrhetic acid mono-glucuronide (GAMG), without producing byproduct glycyrrhetic acid (GA). The results suggest that the purified enzyme may have potential applications in bio-pharmaceutical and biotechnological industry.

1. Introduction

Glycyrrhizin (GL) is the principal active ingredient of liquorice root which has been used as herbal medicine to treat inflammation, infection and many other diseases for several thousand years in China [1]. Chemically, GL is composed of one molecule of glycyrrhetic acid (GA) as aglycon and two molecules of glucuronic acid attached to the C-3 atom of the aglycon moiety (Supplementary data: Fig. S1). By hydrolyzing one terminal glucuronic acid, GL can be transformed into glycyrrhetinic acid monoglucuronide (GAMG), which exhibits much stronger physiological properties as compared to GL [2–6]. GAMG holds a wider spectrum of biological activities than GL, such as anticancer [2,3], anti-anaphylactic [4], antiviral [5], and anti-inflammatory actions [6]. In addition, GAMG possesses a high sugar sweetness equivalent level with an extremely low caloric value, and its sweetness is five times more than that of GL [7,8]. LD_{50} value of GAMG (5000 mg/kg) is much higher than that of GL (805 mg/kg) [9], demonstrating its safeness over GL. Therefore, GAMG is considered to be a new and better food additive and therapeutic agent having much more commercial potential than GL.

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Biotransformation has many advantages over the conventional chemical means such as strict stereo- and region-selectivity, high reaction rate, mild reaction conditions and eco-friendly status. Moreover, it is specifically useful for the structural modification of complex biologically active substances, such as GL. β-Glucuronidase (EC 3.2.1.31) is a glycosidase that catalyses hydrolysis of β-linked glucuronides to yield their various derivatives and free glucuronic acid. β-Glucuronidase has been isolated from many organisms including bacteria [10–12], plants [12,13], animals [14–16] and human [16], but its fungal source remained limited due to restricted screening of this diverse group of organisms [17]. β-Glucuronidase has numerous biotechnological and research applications. As a gene, it has been studied extensively as a positive selection marker for transformed plants, bacteria and fungi carrying glucuronidase gene [18,19]. As a tool enzyme, it is widely versatile and has been used extensively for the structural investigations of proteoglycans and for research purposes in diagnostic research laboratories [20]. Recently, much attention has been paid to its exploitation as a biocatalyst for the transformation of value products. β-glucuronidases from animal livers [7,21], human intestinal bacteria [12,16,22] and yeast *Cryptococcus magnus* MG 27 [23] have been used for the biotransformation of GL. However, most of them exhibit very low hydrolytic selectivity and less activity, which limit their large scale application in the biotransformation

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of GL to synthesize a high valued product such as GAMG. Therefore, there is a need to explore novel sources of β-glucuronidase for the highly selective hydrolysis of GL to maximize the yield of GAMG.

We have recently been focusing our research on filamentous fungi as a source of new β-glucuronidases. During the screening of β-glucuronidase producing organisms, we found a filamentous fungus *Penicillium purpurogenum* Li-3 which produces a specific β-glucuronidase with selective glycyrrhizin-hydrolyzing properties [24]. The objective of the present study was to purify this unique β-glucuronidase produced by *P. purpurogenum* Li-3 and to study the influence of other physical and chemical parameters on its activity. To the best of our knowledge, this is the first report on purification and characterization of a highly selective glycyrrhizin-hydrolyzing β-glucuronidase from filamentous fungi.

2. Materials and methods

2.1. Materials

Glycyrrhizin (GL) and glycyrrhetic acid (GA) were purchased from Sigma Chemical Co. (USA). Glycyrrhetinic acid monoglucuronide (GAMG) was generously donated by Nanjing University of Technology (China). 4-Nitrophenyl-β-D-glucuronide (*p*NPG) was purchased from Sigma Chemical Co. Glycyrrhizin monoammonium salt and all other chemicals used were of analytical grade and were purchased from Merck, China. DEAE-cellulose DE-52 and Sephadex G-100 were from Phamarcia Co. *P. purpurogenum* Li-3 was obtained from the laboratory of Microecology and Biotransformation, Beijing Institute of Technology, Beijing, China. All other chemicals used were of the highest purity and commercially available. All reagents were prepared in Milli Q water (Millipore, USA).

2.2. Medium preparation and enzyme extraction

Mycelial fungus *P. purpurogenum* Li-3 was grown in synthetic medium (500 mL) containing (g/L) glycyrrhizic acid ammonium salt, 20; NH_4NO_3, 3; KH_2PO_4, 0.8; KCl, 0.5; $MgSO_4$, 0.5 as reported earlier [24]. The organism was grown at a temperature of 30 °C, agitation of 180 rpm for 120 h. Fungal mycelium was filtered through Whatmann filter paper (24 mm) and washed thoroughly with distilled water to remove the culture medium. The washed mycelium was macerated in 0.1 M sodium acetate buffer (pH 5.0) by using glass beads in the bead beater (Biospec, Bartlesville, Okla) at 4 °C. The extract was centrifuged at 12,000 × g for 30 min; the supernatant was collected as a source of intracellular enzyme and stored at −20 °C for further use.

2.3. β-Glucuronidase activity assay

β-Glucuronidase activity was determined by measuring the amount of *p*-nitrophenol released from *p*-nitrophenyl-β-glucuronide (*p*NPG). These assays were done at 37 °C, using a mixture (50 μL) containing the enzyme, 1.25 mM substrate, and 50 mM acetate buffer, pH 5.0. Reactions were terminated by the addition of 0.4 M Na_2CO_3 (200 μL), and were monitored at 405 nm for the liberated *p*-nitrophenol. Here one unit of enzyme activity was defined as the amount of enzyme which liberates 1 mmol of *p*-nitrophenol per minute. Protein concentration was estimated by the method of Lowry et al. with bovine serum albumin as standard [25].

2.4. Purification of β-glucuronidase

During the time course of 120 h of incubation, the amount of β-glucuronidase increased rapidly after 40 h and the maximum β-glucuronidase activity was recorded after 96 h (approx. 3.39 U/mg protein) (Fig. 1). Therefore, the fungal mycelium obtained after 96 h of growth was used for the purification of enzyme.

All purification steps were carried out at 4 °C, unless otherwise specified.

2.4.1. DEAE-cellulose DE-52 ion exchange chromatography

The proteins in the crude extract were concentrated by ultrafiltration using an Amicon system with a 10 kDa cut-off membrane. The concentrated extract was then brought to 30% saturation with $(NH_4)_2SO_4$, left for 2 h, and the precipitate formed was removed by centrifugation. The supernatant was then brought to 70% saturation with $(NH_4)_2SO_4$, left for 2 h, and centrifuged. The precipitate was dissolved in a small volume of 20 mM Tris–HCl buffer, pH 8.0, and dialyzed overnight against the buffer.

The dialyzed enzyme solution was applied to DEAE-cellulose DE-52 column (1.6 cm × 20 cm). The column was pre-equilibrated with 20 mM Tris–HCl buffer, pH 8.0, then washed with the same buffer and eluted with 0.0, 0.2, 0.3, 0.4, 0.5 M/L NaCl (500 mL, respectively), at a flow rate of 12 mL/h.

2.4.2. Sphadex G-100 gel filtration

β-Glucuronidase from DEAE-cellulose DE-52 was further purified by a Sephadex G-100 column (1.6 cm × 100 cm) eluting with 100 mM acetate buffer (pH 5.0) at a flow rate of 6 mL/h. The highly active β-glucuronidase fractions were pooled, concentrated by lyophilization and used as purified enzyme for the subsequent studies.

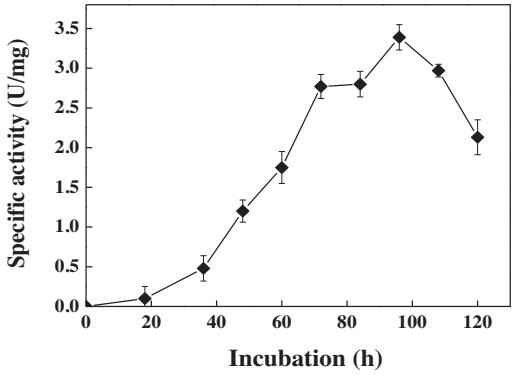

Fig. 1. Production of intracellular β-glucuronidase by *P. purpurogenum* Li-3 growing in medium with 20 g/L glycyrrhizin as the sole carbon source. Results represents means of three experiments, and error bars indicates ±SD.

2.5. Determination of molecular weight

The molecular weight of the enzyme was determined by SDS-PAGE, gel filtration chromatography, and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF). SDS–PAGE was performed according to the method of Laemmli (1970), using a 12% polyacrylamide gel. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue (CBB) R-250. Gel filtration chromatography was performed by using a HiLoad 16/60 Superdex 200 column at a flow rate of 1.0 mL/min for the estimation of molecular weight of the enzyme. The molecular mass was also determined by MALDI-TOF, using Bruker Ultraflex MALDI-TOF/TOF mass spectrometer equipped with a 337 nm nitrogen laser. The matrix was prepared in deionized water containing sinapinic acid (10 mg/ml), 50% acetonitrile and 0.1% TFA. L-Asparaginase was mixed with matrix (1:1) and 2 μL of the sample was spotted onto a well of sample plate, dried at room temperature and then analyzed.

2.6. Effect of pH and temperature

Optimal pH for the enzyme activity was determined by the measurement of residual enzyme activity in 50 mM sodium acetate buffer (pH 3.0–6.0), Tris–HCl buffer (pH 7.0–9.0), and carbonate buffer (pH 10.0–11.0). The pH stability of the enzyme was estimated by measurement of the remaining β-glucuronidase activity after incubation for 1 h in different pH reaction mixtures at 25 °C. The optimum temperature of enzyme was determined by measuring the enzyme activity in 50 mM Tris–HCl buffer (pH 6.0) over a temperature range of 25–70 °C. And the thermal stability of the enzyme was detected by incubating the enzyme for 1 h at various temperatures (25–70 °C) in 50 mM Tris–HCl buffer (pH 6.0).

2.7. Effect of metal ions and reagents on β-glucuronidase activity

The effect of various metal ions and reagents like EDTA and 2-mercaptoethanol on enzyme activity was examined by incubating a mixture consisting 100 μL of the enzyme solution and 100 μL of metal ions or reagents (final concentration, 1 mM) for 20 min at 40 °C, and enzyme activity was then assayed under standard conditions. Enzymatic activities were expressed as relative values (%) with reference to the activity of the enzyme without any metal ion or reagent. The assays were performed in triplicate.

2.8. Enzyme kinetics

The Michaelis constant (K_m), maximal velocity (V_{max}) and turnover numbers (k_{cat}) of the purified enzyme were determined using *p*NPG and glycyrrhizin as substrates in the range of 0.1–10 mM under the optimal assay conditions. The kinetic data were calculated from Lineweaver–Burk plots using the Michaelis–Menten equation. k_{cat} and specificity constants (k_{cat}/K_m) were calculated on the basis of one active site per 69.72 kDa subunit. The assays were performed in triplicate.

2.9. Hydrolysis of glycyrrhizin

Five micrograms of purified enzyme and 100 mg of GL were incubated in 200 μL acetate buffer (10 mM, pH 6.0) at 40 °C. Aliquots (50 μL) taken after 6 h and 15 h were centrifuged and then the concentrations of GL, GAMG and GA were determined by HPLC. The chromatographic conditions were as follows: ODS column (Shim-pack, VP-ODS, 4.6 mm × 250 mm, Shimadzu Corporation, Kyoto, Japan); UV detector; detection wavelength 254 nm; flowrate 1.0 mL/min; mobile phase, water

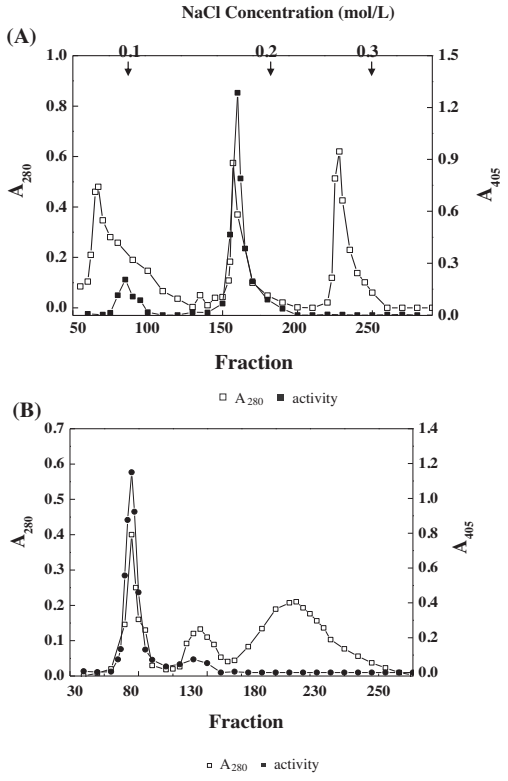

Fig. 2. Elution profile for purification of *P. purpurogenum* β-glucuronidase on (A) DEAE-cellulose DE-52 ion exchange chromatography. (B) Elution profile on Sephadex G-100 chromatography column. Fractions were monitored at 280 nm for protein (□) and assayed for enzyme activity (■).

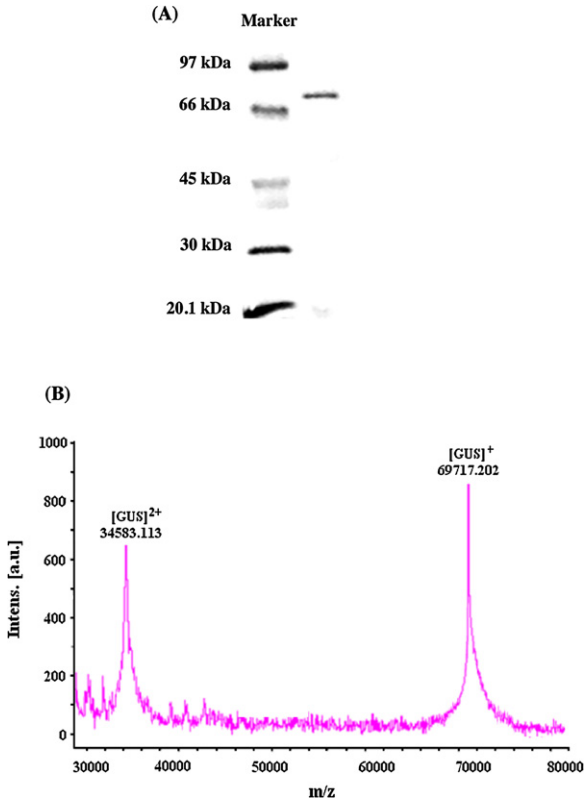

Fig. 3. (A) SDS-PAGE of the purified β-glucuronidase from *P. purpurogenum* Li-3 (right lane) and molecular weight markers (left lane). The markers were phosphorylase b (97 kDa), BSA (66 kDa), egg ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20.1 kDa), all from GE Healthcare. The right lane was *P. purpurogenum* β-glucuronidase with a molecular mass of 69 kDa. (B) Mass spectrometry of β-glucuronidase (GUS) by MALDI-TOF.

(pH 2.85 with 0.6%, v/v, acetic acid) and methanol at 19:81 (v/v); injection volume 10 μL. The retention times for GL, GAMG and GA were around 5.77, 13.73 and 21.88 min, respectively (Fig. 5A).

3. Results and discussion

3.1. Production and purification of β-glucuronidase

The enzyme purification protocol is summarized in Table 1. The β-glucuronidase was purified 80.6-fold with 26.8% yield from the crude enzyme extract. First, the proteins in the crude extract were concentrated three fold by ultrafiltration using an Amicon system with a 10 kDa cut-off membrane. Then, the retentates were precipitated with 30–70% $(NH_4)_2SO_4$ which achieved 7.8-fold enzyme purification and 63.1% enzyme was recovered. Then, the concentrated enzyme preparation was fractionated by anionic exchange chromatography by using a DEAE-cellulose DE-52 column. Fig. 2A showed the main elution peaks correspond to three NaCl concentrations of 0.1, 0.2 and 0.3 M. Two activity peak eluted in 0.1 and 0.2 M NaCl were pooled for further purification. The remaining protein contaminants were removed by gel filtration using Sephadex G-100 (Fig. 2B). The fractions with the maximum enzyme activity were collected and examined further for purity by gel electrophoresis.

The specific activity of β-glucuronidase purified from *P. purpurogenum* Li-3 was higher (272 U/mg) compared with the enzyme purified from *Streptococcus* LJ-22 (137 and 190 U/mg) by Park et al. [10] or β-glucuronidase from *Aspergillus niger* (29.3 U/mg) [20] and *Escherichia coli* HGU-3 (17.78 U/mg) [26].

3.2. Homogeneity and molecular mass of β-glucuronidase

β-Glucuronidase from *P. purpurogenum* Li-3 appeared as a single band with a molecular weight of 69 kDa in SDS–PAGE analysis (Fig. 3A), which was consistent with the molecular mass (69.72 kDa) estimated by MALDI-TOF MS analysis (m/z = 69,717) (Fig. 3B). Gel filtration studies elucidated that the protein holds a native molecular mass of about 145.2 kDa. These results indicated that the enzyme is a dimeric protein with two identical subunits similar to other β-glucuronidase from *Streptococcus* LJ-22 [10]. Different molecular weights of β-glucuronidases from various sources have already been reported, such as 43 kDa for *Eubacterium* sp. GLH [22], 55–58 kDa for *Scutellaria* root [12,13], 67 kDa for *Streptococcus* LJ-22 [10], 68 kDa for *A. niger* [20], 74 kDa for mouse epididymis [14] and 90 kDa for *Pomacea* sp. [15]. The different in molecular weights of various β-glucuronidases from different sources could be due to the genetic variations present among different species which play important roles in determining the size, shape and molecular mass of such enzymes. Generally, β-glucuronidases have been reported to have molecular masses between 50 and 70 kDa.

3.3. Effect of pH on β-glucuronidase activity and stability

The effects of pH on the activity of purified β-glucuronidase from *P. purpurogenum* Li-3 are shown in Fig. 4A. The optimal pH for GL hydrolysis was 6.0. A marked loss in activity was found at pH 3.0. Moreover, a considerable decrease in activity was also observed at alkaline pH. The optimal pH of β-glucuronidase vary greatly according to their source as β-glucuronidase from *A. niger* exhibited an optimal pH of 3.0 [20], while β-glucuronidase from *Scutellaria*

Table 1
Purification of β-glucuronidases from *P. purpurogenum* Li-3.

Purification steps	Total protein (mg)	Total activity ($\times 10^3$ U)	Specific activity (U mg^{-1})	Recovery (%)	Purification (fold)
Crude extract	1206	4.08	3.39	100	1.0
Ultrafiltration	438	3.65	8.34	89.5	2.5
$(NH_4)_2SO_4$ precipitation	97.7	2.57	26.4	63.1	7.8
DEAE-cellulose DE-52	10.2	1.80	177	44.2	52.3
Sephadex G-100	4.05	1.09	273	26.8	80.6

root showed maximal activity at pH 4.7 [12]. The optimal pH of both baicalin-β-glucuronidase and *Eubacterium* β-glucuronidase was found to be 5.0 [11,13]. β-Glucuronidase from *Streptococcus* LJ-22 displayed maximal activity at pH 6.5 and 7.0 [10]. However, β-Glucuronidase from callus cultures of *Scutellaria baicalensis* Georgi was more active at alkaline pH with optimal pH from 7.0 to 8.0 [27]. The differences in optimal pH might be associated with difference in intracellular pH of species producing β-glucuronidase, as well as differences in substrates or assay conditions used.

The effects of pH on the stability of β-glucuronidase from *P. purpurogenum* are also shown in Fig. 4A. The purified enzyme was found to be quite stable within a pH range of 5.0–8.0 at 40 °C for 1 h (Fig. 4A). Zhang et al. [13] reported that baicalin-β-glucuronidases exhibited high stability over a pH range from slightly acidic to neutral (pH 4.0–7.0). Kuroyama et al. [20] reported that a β-glucuronidase from *A. niger* was stable within pH ranges of 4.5–9.0. Therefore, the stability property of *P. purpurogenum*

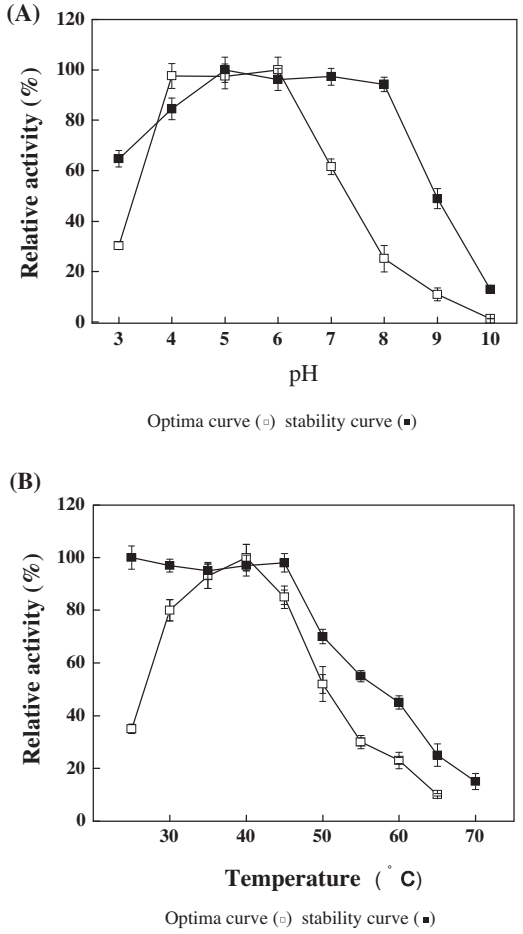

Fig. 4. (A) Effect of pH on the activity and stability of the purified β-glucuronidase. (B) Effect of temperature on the activity and stability of the purified β-glucuronidase. The relative activities were defined as the percentage of the maximum activity detected in the assay. Results represents means of three experiments, and error bars indicates ±SD.

β-glucuronidase could be exploited in the processes that require large pH changes.

3.4. Effect of temperature on the activity and stability of β-glucuronidase

The optimum temperature of the purified enzyme was determined by varying the reaction temperature. As it can be observed in Fig. 4B, the enzyme was active at temperatures ranging between 30 and 45 °C, exhibiting maximal activity at 40 °C. The same optimal temperature (40 °C) of β-glucuronidase from *A. terreus* [28] was also reported. However, *Scutellaria* root β-glucuronidase showed the optimal temperature at 50 °C [13].

Thermal stabilities of purified β-glucuronidase from *P. purpurogenum* is also depicted in Fig. 4B. β-Glucuronidase was quite stable up to 45 °C with a residual activity of 95–99% incubating at pH 6.0 for 1 h. A decrease in activity was observed when heated at temperatures above 50 °C. At high temperatures, β-glucuronidase most likely underwent denaturation and lost its activity. The effect of incubation time on enzyme activity was also examined at 40 °C and pH 6.0 for the purified β-glucuronidase. Results showed the purified enzyme maintained 93.4% of its original activity after 10 h at 40 °C and pH 6.0, after 20 h, the enzyme still maintained about 85.7% of its activity. Similar thermal stability was reported for β-glucuronidase from *Aspergillus terreus* [28]. Kuroyama et al. [20] reported that β-glucuronidase from *A. niger* retained its full activity up to 60 °C. Zhang et al. [13] reported that baicalin-β-glucuronidase from *Scutellaria* roots was found stable up to 70 °C. In general, thermal stability of β-glucuronidase varies with species as well as with incubation time.

3.5. Effect of various metal ions and reagents on enzyme activity

The sensitivity of purified β-glucuronidase from *P. purpurogenum* Li-3 to various metal ions was tested, and the results are depicted in Table 2. Mg^{2+} ions (at concentrations of 10 mM) significantly enhanced β-glucuronidase activity (about 1.33-fold). Similar

Table 2
Effect of various metal ions and chatropic agents on the activity of β-glucuronidase from *P. purpurogenum* Li-3.

Metal ions	Concentration	Relative enzyme activity (%)
None		100.0
Ca^{2+}	10 mM	100 ± 3.9
Cu^{2+}	1 mM	3.2 ± 0.2
Hg^{2+}	1 mM	10.9 ± 0.5
Fe^{2+}	10 mM	76.3 ± 2.2
Co^{2+}	10 mM	84.9 ± 2.5
Mg^{2+}	10 mM	133 ± 3.8
Ni^{2+}	10 mM	95.6 ± 1.3
Zn^{2+}	10 mM	24.7 ± 2.1
Mn^{2+}	10 mM	30.2 ± 3.3
Al^{3+}	10 mM	100 ± 2.4
2-Mercaptoethanol	10 mM	83.5 ± 4.4
EDTA	10 mM	89.7 ± 2.1
Urea	10 mM	75.4 ± 3.1
SDS	0.5% (w/v)	87.6 ± 2.4
Triton X-100	0.5% (v/v)	88.5 ± 1.8

Table 3
Kinetic parameters of β-glucuronidase from *P. purpurogenum* Li-3 reacting with two glucuronide compounds.

Substrate	K_m (mM)	V_{max} (mmol mg^{-1} min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
*p*NPG	1.84	285.7	1.66×10^5	9.02×10^4
Glycyrrhizin	0.33	59.0	3.42×10^4	1.04×10^5

results were reported for baicalin-β-glucuronidase [13] and β-glucuronidase from *Pomacea* sp. [15]. Ca^{2+} and Al^{3+} did not show any considerable effect on enzyme activity, while heavy metal such as Cu^{2+}, Hg^{2+}, Zn^{2+}, Mn^{2+}, Ni^{+} and Co^{2+} exhibited different inhibitory effects on the enzyme activity, in particular Cu^{2+} ions (at a concentrations of 1.0 mM) which caused the highest decrease in enzymatic activity (3.2% remaining, Table 2). Similar results were also reported in the literature for other β-glucuronidases. Both β-glucuronidases from *Scutellaria* root [13] and *Streptococcus* LJ-22 [10] were significantly inhibited by Cu^{2+}, β-glucuronidase from *A. niger* was inhibited by Hg^{2+} [20]. The salts of heavy metals such as Cu^{2+} and Hg^{2+} may interact with thiol groups of enzyme thereby causing conformational changes in enzyme. Many enzymes contain thiol (—SH), alcohol, or acid groups as part of their active sites, and binding of any unwanted substance with these groups could cause the irreversible inhibition of enzyme activity by covalent modification of the enzyme structure [29].

The influence of some reagents on the activity of the enzyme purified from *P. purpurogenum* Li-3 is also displayed in Table 2. In the presence of SDS (0.5%), the purified enzyme showed almost 87.6% residual activity, while in the presence of urea and EDTA (10 mM), the purified enzyme lost approximately 24.6 and 10.3% activity, respectively. The purified enzyme was also susceptible to the nonionic detergent Triton X-100 and reducing agents 2-mercaptoethanol and its activity was reduced by 11.5% and 12.5%, respectively.

3.6. Kinetic parameters

Kinetic data for purified β-glucuronidase from *P. purpurogenum* Li-3 are summarized in Table 3. The effect of varying the substrate concentration on the reaction rate was investigated by using 4-nitrophenyl-β-D-glucuronide (*p*NPG) and glycyrrhizin (GL) as substrates. K_m and V_{max} for the hydrolysis of *p*NPG were 1.84 mM and 285.7 mmol mg^{-1} min^{-1}, respectively, and the corresponding values for the hydrolysis of GL were 0.33 mM and 59.0 mmol mg^{-1} min^{-1}, respectively. K_m is often associated with the affinity of the enzyme for substrate [30]. The result indicated that β-glucuronidase from *P. purpurogenum* Li-3 had a higher affinity for GL than *p*NPG. The variations in the affinity value of various glucuronides having different aglycone reflects that the aglycone group of the substrate has a profound effect on the K_m value. The K_m value of the purified β-glucuronidase produced by the *Eubacterium* sp. GLH from the human intestine was 77 mM [22]. This suggests that the β-glucuronidase produced by filamentous fungus *P. purpurogenum* Li-3 possesses higher affinity for GL compared to the β-glucuronidase produced by intestinal bacteria. The catalytic efficiencies (k_{cat}/K_m) for the hydrolysis of *p*NPG and GL were calculated to be 9.02×10^4 and 1.04×10^5 s^{-1} mM^{-1}, respectively. The k_{cat}/K_m value of enzyme for *p*NPG was lower than that of GL, indicating the fact that β-glucuronidase from *P. purpurogenum* Li-3 holds a much higher catalytic efficiency for GL as compared to *p*NPG.

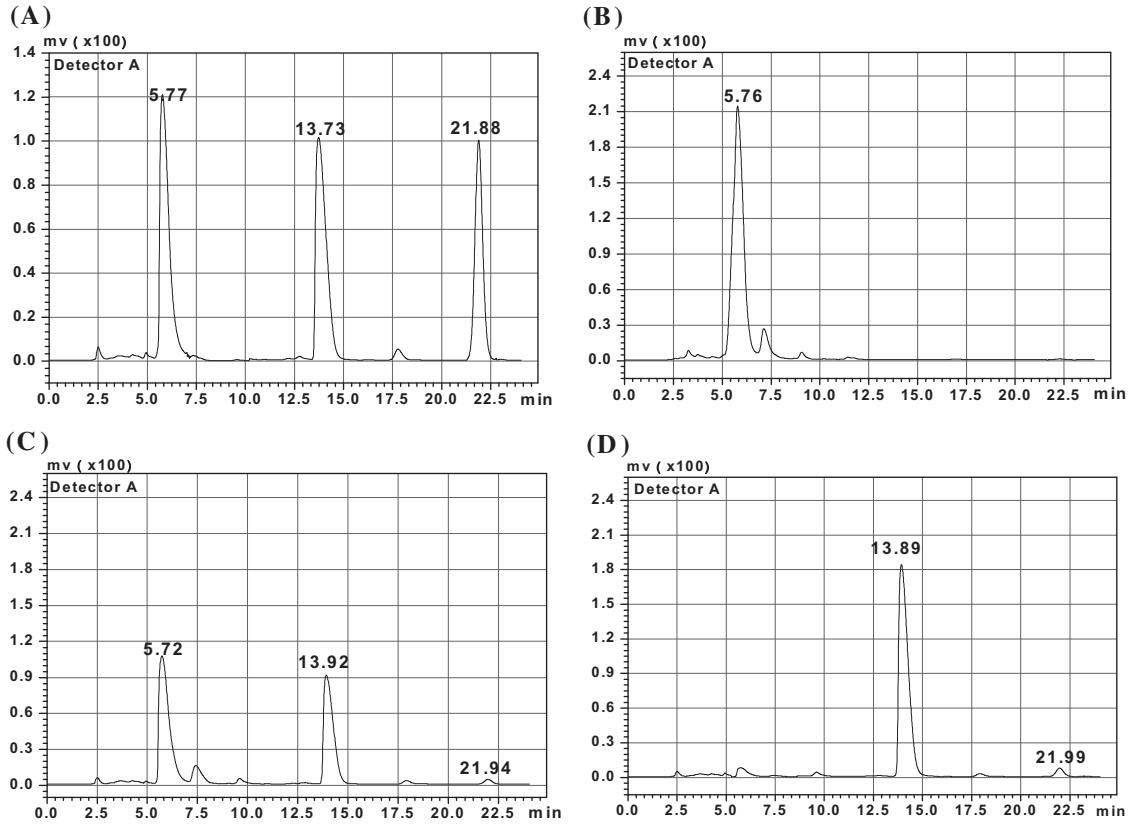

Fig. 5. Hydrolysis assay. Hydrolysis reaction of GL was carried out by incubating the purified β-glucuronidase for 0 h (B), 5 h (C) and 15 h (D). Standards samples of GL (5.7 min), GAMG (13.7 min) and GA (21.8 min) were run as control (A).

3.7. Hydrolysis of glycyrrhizin by purified β-glucuronidase

During the reaction course of glycyrrhizin hydrolysis by purified β-glucuronidase, it was found that at time 0 h of incubation, a single peak (5.76 min) of glycyrrhizin was identified (Fig. 5B). After 6 h, the peak of glycyrrhizin became small and a new peak of GAMG at 13.92 min was visible (Fig. 5C). After 15 h, only a single and bigger peak corresponding to GAMG at 13.89 min was obtained (Fig. 5D). This result suggests that the purified enzyme possessed high chemical bond selectivity and could transform GL into GAMG, without any formation of byproduct of GA. The maximum yield of GAMG was estimated around 95.5% in 15 h under experimental conditions as calculated by the peak areas taken from HPLC chromatogram. Wang et al. reported an *Aspergillus parasiticus* β-glucuronidase which could transform GL into GA, but no GAMG was produced [31]. The lysosomal β-glucuronidase from animal livers [7] and human intestinal bacteria [16,22,32] could convert GL to GAMG, but GA was produced as a byproduct. The selective hydrolysis of GL by β-glucuronidases from different sources has been reported for the production of GAMG. Kuramoto et al. [23] described a β-glucuronidase from yeast *C. magnus* MG 27 for the direct conversion of GL into GAMG. Park et al. [10] also isolated a β-glucuronidase from bacteria *Streptococcus* LJ-22 having capability of highly selective hydrolysis of GL. However, this is the first report on the purification and characterization of a highly selective glycyrrhizin-hydrolyzing β-glucuronidase from filamentous fungi.

4. Conclusion

We have shown that the fungus *P. purpurogenum* Li-3 produced a highly selective glycyrrhizin-hydrolyzing β-glucuronidase. This is the first report on the purification of β-glucuronidase from *Penicillium* species. The purified enzyme was found to be composed of two identical subunits with a molecular mass of 69.72 kDa. The activity of the enzyme was enhanced by Mg^{2+} ions. The enzyme was quite stable within a large pH and temperature range. The results suggest that this purified β-glucuronidase can be considered as an appropriate and excellent biocatalyst for the biosynthesis of GAMG in bio-pharmaceutical and biotechnological industry.

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