Process Biochemistry 48 (2013) 358–363

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

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

Shuping Zou[b,1], Guiyan Liu[a,1], Imdad Kaleem[a], Chun Li[a,*]

[a] School of Life Science, Beijing Institute of Technology, Beijing 100081, People's Repubic of China
[b] Institute of Bioengineering, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China

ARTICLE INFO

Article history:
Received 13 August 2012
Received in revised form
16 December 2012
Accepted 17 December 2012
Available online 25 December 2012

Keywords:
Penicillium purpurogenum
β-Glucuronidase
Purification
Glycyrrhizin
Selective hydrolysis

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.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2012.12.008.

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

* Corresponding author. Tel.: +86 10 68913171; fax: +86 10 68913171.
E-mail addresses: lichun@bit.edu.cn, lichun04@bit.edu.cn (C. Li).
[1] These authors contribute to this work equally.

1359-5113/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.procbio.2012.12.008

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.

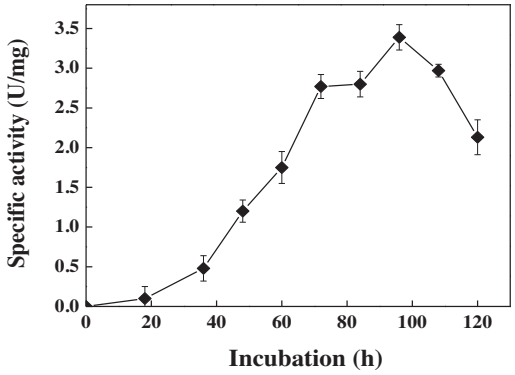

Fig. 1. Production of intracellular β-glucuronidase by *P. purpurogenum* Li-3 growing in medium with 20 g/L glycyrrhizin as the sole carbon source. Results represents means of three experiments, and error bars indicates ±SD.

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