Journal of Molecular Catalysis B: Enzymatic 57 (2009) 278–283

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

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

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ARTICLE INFO

Article history:
Received 17 April 2008
Received in revised form
26 September 2008
Accepted 27 September 2008
Available online 14 October 2008

Keywords:
Purification
Properties
β-Glucosidase
Fusarium proliferatum
Ginsenoside Rg_3
Ginsenoside Rh_2

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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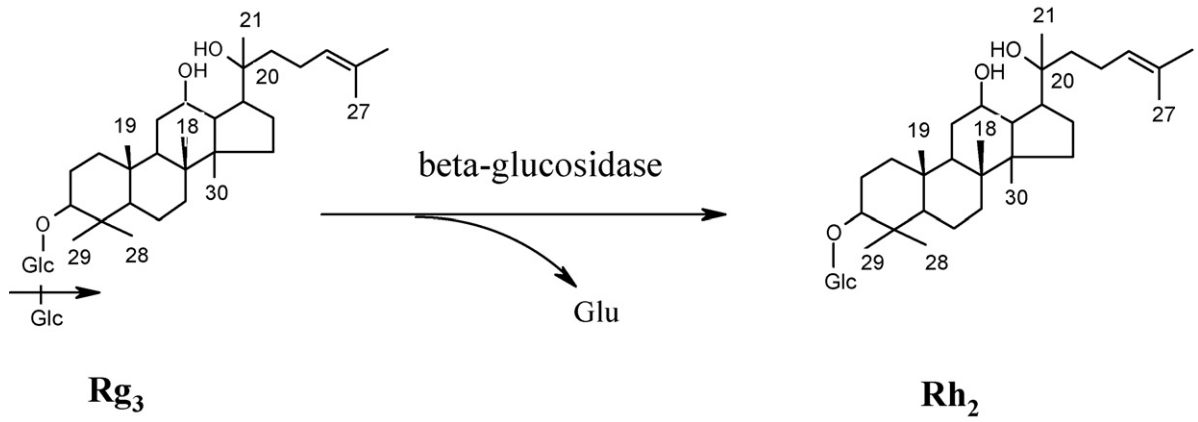
doi:10.1016/j.molcatb.2008.09.017



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-