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Purification and characterization of a glycosidase with hydrolyzing multi-3-*O*-glycosides of spirostanol saponin activity from *Gibberella intermedia*

Hui Li, Xiangdong Wang, Yang Ma, Nannan Yang, Xiaojuan Zhang, Zhenghong Xu, Jinsong Shi *

School of Pharmaceutical Science, Jiangnan University, Wuxi 214122, PR China

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ABSTRACT

The strain *Gibberella intermedia* WX12 preserved in our lab showed a strong ability to degrade dioscin from *Dioscorea Zingiberensis* C. H. Wright into diosgenin. The glycosidase enzyme of this strain was purified by a procedure consisting of ammonium sulfate fractionation, anion-exchange and hydrophobic chromatography and was named GiGly. This enzyme was a monomer with molecular mass of approximately 45 kDa. The optimal temperature and pH were 50 °C and 8.0, respectively. GiGly was stable in the pH range of 5.0–8.0 and retained 80% of its original activity at pH 7.0 for 12 h. In the presence of metal ions including Na^+, K^+ and Mg^{2+} slightly increased GiGly activity, and Fe^{2+}, Fe^{3+}, Cu^{2+} and Mn^{2+} notably inhibited the activity. Meanwhile, GiGly showed high substrate specificity for multi-3-*O*-glycosides of spirostanol saponins such as dioscin, trillin and polyphyllin VII, and was inactive toward the substrates with terminal groups of rhamnosyl, glucosyl, galactosyl of ginsenoside Re and saikosaponin A. GiGly from *G. intermedia* WX12 could only hydrolyze glycosidic bonds at the C-3 position on steroidal saponins, which have similar structure with dioscin, and can be transformed into diosgenin.

1. Introduction

Dioscorea Zingiberensis C. H. Wright (DZW) is an important natural resource of diosgenin (25R-spirost-en-3β-ol), which is widely used for commercial synthesis of sex hormones and corticosteroids [1–3]. Recently, many investigations have been reported about the pharmacological attributes of diosgenin and its physiologically active properties, such as improving cardiovascular function, anticancer function, antagonistic effect on rheumatoid arthritis, and antimalarial action [4–7]. Diosgenin exists as a form of glycosidal steroidal saponin in DZW. In the steroid production industry, the extraction of diosgenin from DZW tubers is predominantly based on acid hydrolysis followed by petroleum extraction, resulting in the release of high-acid and high-strength wastewater into the ecosystem [8,9].

According to previous reports, microorganisms or enzymes could extract diosgenin from DZW, which is an environmental-friendly alternative compared with acid hydrolysis method [5,10]. The biotransformation method uses mild reaction conditions, high selectivity and cleaner production processes [11]. However, the enzyme method for hydrolyzing the raw material from DZW to produce diosgenin is not economical because commercial enzymes have low efficiency and are expensive [12]. Many strains including *Curvularia lunata, Penicillium melinii*, *Aspergillus niger*, *Aspergillus fumigates*, *Absidia* sp.d38 and *Rhizopus* sp. have been identified to selectively hydrolyze the glycosyl residue of steroidal saponins at C-3 position [13–15].

Recently, some glycosidases, such as glucoamylase from *C. lunata* [16], dioscin-α-L-rhamnosidase from animal liver [17], β-glucosidase from *A. fumigates* [14], and dioscin-glycosidases from *A.* sp.d38 strain, were purified and characterized [15]. However, most are restricted in their substrate spectrum and do not yield diosgenin as the final product. *C. lunata* is able to hydrolyze terminal rhamnosyls that are linked end-rhamnosyl residues at C-3 position of steroidal saponins, such as polyphyllin III and polyphyllin VII [13,16,18]. The dioscin-α-L-rhamnosidase from pig liver hydrolyzed two rhamnosides of dioscin to diosgenyl-*O*-β-D-Glc, showing that the enzyme has high specificity [17].

* Corresponding author at: School of Pharmaceutical Science, Jiangnan University, 1800 Lihu Avenue, 214122 Wuxi, China.
E-mail address: shijs@163.com (J. Shi).

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Dioscinase from the microorganism sp.s00c strain hydrolyzes 3-*O*-α-L-(1 → 2)-Rhap and 3-*O*-α-L-(1 → 4)-Rhap from dioscin to produce 3-*O*-β-D-glucopyranosyl-diosgenin, and also hydrolyzes 3-*O*-α-L-(1 → 2)-Rhap and 3-*O*-β-L-(1 → 3)-Glcp from 3-*O*-α-L-(1 → 2)-Rhap, [3-*O*-β-L-(1 → 3)]-β-Glcp-diosgenin to produce 3-*O*-β-D-glucopyranosyl-diosgenin [19]. In addition, the enzyme (dioscin-glycosidase) isolated from *Absidia* strain was capable of yielding aglycone as the final product [15]. Meanwhile, with the catalyzation of β-glucosidase, purified from a newly isolated strain of *A. fumigatus*, various spirostanosides can be transformed to diosgenin [14].

In our previous research, a fungal strain (*G. intermedia* WX12) was isolated and had the ability to hydrolyze dioscin from DZW into diosgenin [20]. To better understand GiGly properties, this work focused on the purification and characterization of GiGly produced by the strain *G.intermedia* WX12. The molecular weight and substrate specificity of the purified enzyme were also characterized. This work intends to establish the foundation for the green production of diosgenin from DZW.

2. Experimental

2.1. Materials

The standards of *p*-nitrophenyl-β-D-glucopyranoside (pNPG) and *p*-nitrophenol were from Sangon Biotech, China. The standards of dioscin ([3-*O*-α-L-(1 → 4)-Rha, 3-*O*-α-L-(1 → 2)-Rha]-β-D-Glc-diosgenin]), trillin ([3-*O*-α-L-(1 → 4)-Ara, 3-*O*-α-L-(1 → 2)-Rha]-β-D-Glc-diosgenin]), polyphyllin VII, Saikosaponin A and Ginsenoside Re (Fig. 1), with 98% purity, were from Shanghai Shifeng Biological Technology Co., Ltd., China. Diosgenin (98% purity) was from Sinopharm Chemical Reagent Co., Ltd., China.

2.2. Strain and culture conditions

G. intermedia WX12 was stored in the China General Microbiological Culture Collection Center (No 5901; Beijing, China). The *G. intermedia* WX12 strain was cultured on a rotary shaker at 220 rpm, 30 °C in flasks (250 mL) with 30 mL of fermentation medium (g/L): glucose 20, yeast 5, NaCl 1.16 and KH_2PO_4 2.72 at pH 7.0 for 96 h. The enzyme activity and biomass were measured during the fermentation.

2.3. Enzyme extraction and purification

The fermented broth (300 mL) was centrifuged to obtain the cells which were ground with quartz sand and extracted with buffer (20 mM PBS, pH 6.0), and mixed with the supernatant as the crude enzyme. $(NH_4)_2SO_4$ was then slowly added to the cell-free culture which was concentrated by ultrafiltration with stirring to 60% saturation and the mixture stored at 4 °C for 4 h. After removing the protein precipitated by the centrifugation, more $(NH_4)_2SO_4$ was added to 90% saturation and the mixture stored at 4 °C overnight. Thereafter, the mixture was centrifuged to harvest the crude enzyme, which was then dialyzed against 20 mM phosphate buffer (pH 6.0). The non-dissolved fraction was removed by centrifugation, and the resulting crude enzyme solution subjected to further enzyme purification.

Ten ml of the crude enzyme solution were loaded on an anion-exchange column of Q-Sepharose Fast Flow (2.6 cm × 30 cm, Pharmacia), which had been equilibrated with 20 mM phosphate buffer (pH 6.0) in advance, and the proteins were fractionated stepwisely with 0.1 M–2 M NaCl at a flow rate of 2 mL/min. The eluted fractions were collected every 10 mL and assayed for the enzyme activity. The main active fractions, which eluted at 0.2 M NaCl were pooled. The active enzyme solution eluted at 0.2 M NaCl was dialyzed aginst 20 mM PBS (pH 7.2, with 1 M $(NH_4)_2SO_4$).

The active fraction were then applied to a Phenyl-Sepharose 6 Fast Flow column (2.6 × 30 cm, GE Healthcare), which had been equilibrated with 1 M $(NH_4)_2SO_4$ in 20 mM phosphate buffer (pH 7.2) and eluted with 20 mM phosphate buffer (pH 7.2). The purified enzyme possessing the highest activity was collected for further investigation.

2.4. Assays of enzymatic activity

The enzyme activity was measured using pNPG as substrate [16]. The reaction mixture containing 1.0 mL phosphate buffer (20 mM, pH 6.0), 0.5 mL pNPG (1.0 mM) and 0.5 mL enzyme solution was incubated at 40 °C for 30 min. The reaction was stopped by adding 1 mL Na_2CO_3 (0.5 M) and the absorption was measured at 400 nm. One unit (U) of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1.0 μmol *p*-nitrophenol per minute.

2.5. Estimation of enzyme molecular mass

The purity and molecular mass of the purified enzymes were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis was carried out on a 5% (w/v) stacking gel and a 12% (w/v) separating gel. The sample was pretreated with 20 mM β-mercaptoethanol. The gel was stained with coomassie brilliant blue R-250 followed by destaining with anhydrous ethanol–acetic acid–water (2:1:7, v/v/v).

2.6. Analytical methods

Biomass was determined as follows: the broth was centrifuged at 12,000 rpm for 10 min. The precipitated mycelia were then collected and washed twice with distilled water. Washed mycelia samples were dried at 80 °C to constant weight.

TLC and HPLC were used to analyze the enzymatic reaction product. The reaction product in the acetonitrile was analyzed by TLC with chloroform: methanol: H_2O [65:35:10 (v/v/v)] as the developing solvent I with diosgenin as standard. And for the rhamnose and glucose analysis by TLC, the developing solvent II with chloroform: methanol: ethyl acetate: petroleum ether [2:2:1:3 (v/v/v/v)] was used. Visualization was performed using 10% H_2SO_4–EtOH spray reagent, followed by heating. A C-18 column (Agilent TC-C18, 4.6 mm × 150 mm, 5 μm) was used for detecting the product. The measuring wavelength was 203 nm and the injected volume was 20 μL. The mobile phase was A (acetonitrile) and B (water) = 90:10, the flow rate was 1.0 mL/min.

Protein concentration was determined using the Bradford Protein Assay Kit (B302DB0001, Sangon Biotech, China) using bovine serum albumin as the standard, and the staining solution was Coomassie brilliant blue G-250. All experiments were performed in triplicate.

2.7. Substrate specificity

The substrate specificity of the glycosidase was determined by analyzing the products for hydrolysis of different saponins (dioscin, trillin, polyphyllin VII, Saikosaponin A and Ginsenoside Re). Both substrates and glycosidase were prepared with phosphate buffer (20 mM, pH 6.0) and the reaction was performed by incubating 1.0 mL phosphate buffer, 0.5 mL substrate (1 mM) with 0.5 mL GiGly (90 U/mL) at 40 °C for 30 min with shaking. The reaction was stopped by liquid nitrogen before the samples were lyophilized and