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Purification and characterization of a thermostable α-galactosidase with transglycosylation activity from *Aspergillus parasiticus* MTCC-2796

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ABSTRACT

An extracellular thermostable α-galactosidase from *Aspergillus parasiticus* MTCC-2796 was purified 16.59-fold by precipitation with acetone, followed by sequential column chromatography with DEAE-Sephadex A-50 and Sephadex G-100. The purified enzyme was homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). It was found to be a monomeric protein with a molecular weight of about 67.5 kDa. The purified enzyme showed optimum activity against o-nitrophenyl-α-D-galactopyranoside (oNPG) at pH 5.0 and a temperature of 50 °C. The enzyme was thermostable, showing complete activity even after heating at 65 °C for 30 min. The enzyme showed strict substrate specificity for α-galactosides and hydrolyzed oNPG (K_m = 0.83 mM), melibiose (K_m = 2.48 mM) and raffinose (K_m = 5.83 mM). Among metal ions and reagents tested, Ca^{2+} and K^+ enhanced the enzymatic activity, but Mg^{2+}, Mn^{2+}, ethylenediaminetetraacetic acid (EDTA) and 2-mercaptoethanol showed no effect, while Ag^+, Hg^{2+} and Co^{2+} strongly inhibited the activity of the enzyme. The enzyme catalyzed the transglycosylation reaction for the synthesis of melibiose.

1. Introduction

α-Galactosidase (EC 3.2.1.22) is an exoglycosidase that catalyzes the hydrolysis of terminal α-1,6-linked-D-galactose residues from simple galactose-containing oligosaccharides such as melibiose, raffinose, stachyose and verbascose, as well as more complex polysaccharides including galactomannans [1]. α-Galactosidase may have great potential in various biotechnological applications. In order to improve the nutritional value of legume-based food, α-galactosidases can be applied for the reduction or elimination of antinutritive galacto-oligosaccharides (called raffinose family sugars) that cause flatulence [2,3]. In the beet sugar industry, this enzyme is used to remove raffinose from molasses in order to increase the yield of crystallized sugar [4,5]. α-Galactosidase can also be used for the removal of a quantitative proportion of galactose moieties from guar gum in order to improve the gelling properties of the polysaccharide and to make them comparable to those of locust bean gum [6]. Furthermore, galacto-oligosaccharides produced by the transfer reaction of α-galactosidase can be used as a prebiotic in functional food [7]. Apart from this, α-galactosidase is also of medical importance. Type 'B' erythrocytes, which contain 3-O-α-D-galactopyranoside, can be transformed into the more universally transferable type 'O' erythrocytes by exposure to α-galactosidase [8]. In humans, mutations in the X-chromosomal α-galactosidase gene can lead to an accumulation of α-linked galactosides in tissues, resulting in a lysosomal storage disorder called Fabry's disease [9]. Enzyme replacement therapy with α-galactosidase has been introduced as a potential therapeutic option for the treatment of Fabry's patients [10].

Over the last few years, glycosyl hydrolases have gained interest as practical enzymes for the convenient synthesis of biologically relevant glycosidic linkages in the field of oligosaccharide production [11]. So far we have a great deal of knowledge on the production of β-galacto-oligosaccharides using β-galactosidases and lactose as a substrate [12]. Little research, however, has focused on the production of α-galactosidases possessing transglycosylation activity. Therefore, together with the hydrolytic action, we investigated synthetic aspects of the enzyme, in order to establish a simple strategy for the synthesis of α-galacto-oligosaccharides.

α-Galactosidase has been isolated and purified from a variety of sources such as plants, animals and microorganisms. Included among these sources are few fungi [13–17]. As the use of fungal α-galactosidase increases, it becomes important to isolate new fungal strains producing enzymes with properties more amenable to industrial applications [18]. Thermostable enzymes are of greater interest, because they permit processing at elevated temperature (above 60 °C) at which rapid product formation can be achieved without the growth of mesophilic organisms. Most of the thermostable α-galactosidases have been purified and characterized from thermophilic bacteria and fungi [19]. Recently, Shankar et al. [20] reported the purification and characterization of a thermostable α-galactosidase from a mesophilic strain of *Aspergillus*

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terreus. However, reports of thermostable α-galactosidases from mesophilic fungi are very limited. Before now, to the best of our knowledge, no thermostable α-galactosidase from *Aspergillus parasiticus* has been reported in the literature. Hence, we report for the first time the isolation, purification, characterization and transglycosylation activity of a thermostable α-galactosidase secreted by the mesophilic fungus *Aspergillus parasiticus* MTCC-2796.

2. Materials and methods

2.1. Materials

The compounds 4-methylumbelliferyl-α-D-galactoside and oNPG were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Galactose, raffinose, melibiose, yeast extract and all other chemicals used were of analytical grade and were purchased from Merck, India. DEAE-Sephadex A-50 and Sephadex G-100 were from GE Healthcare, U.K. *Aspergillus parasiticus* MTCC-2796 was obtained from the Institute of Microbial Technology, Chandigarh, India.

2.2. Crude enzyme extraction

α-Galactosidase production was carried out in a liquid culture medium, composed of 7.0 g l^{-1} KH_2PO_4, 2.0 g l^{-1} K_2HPO_4, 0.1 g l^{-1} $MgSO_4{\cdot}7H_2O$, 1.0 g l^{-1} $(NH_4)_2SO_4$, 0.6 g l^{-1} yeast extract and 1% (w/v) galactose, at pH 6.5. The 100 ml Erlenmeyer glass flasks containing 25 ml of culture medium were inoculated with fungal spores (10^8 ml^{-1}) and incubated at 30 °C for 24 h in an automatic incubator. After incubation, the culture supernatants were collected by filtration through filter paper (Grade 4B, Sd fine, Mumbai, India). The filtrates were subjected to centrifugation (5000 rpm) for 10 min at 4 °C, and the obtained supernatant was used in the enzyme assay.

2.3. Enzyme and protein assay

The α-galactosidase assay was carried out using a modified version of the method of Garro et al. [21]. The reaction mixture contained 50 μl of 20 mM oNPG, 50 μl McIlvaine buffer (pH 5.0), 100 μl cell-free extract; final volume: 200 μl. The mixture was incubated at 50 °C for 10 min, and the reaction was stopped by adding 3 ml of sodium carbonate (0.25 mM). The liberated o-nitrophenol was quantitated by using a UV/VIS spectrophotometer (Systronics, India) to measure the absorbance of the solution at 415 nm.

The activity against raffinose was assayed for 30 min at 50 °C using a reaction mixture containing 50 μl of McIlvaine buffer, pH 5.0, 100 μl of enzyme extract and 50 μl of 50 mM substrate solution. The amount of reducing sugar was quantitated using the 3,5-dinitrosalicylate method [22]; the activity against melibiose was assayed using the same reaction system, but glucose formation was measured by the glucose oxidase method [23].

One enzyme unit (U) was defined as the amount of enzyme that released 1.0 μmol of product (o-nitro phenol or reducing sugar) per minute under the given assay conditions. The results are expressed as U/ml. Protein concentration was determined by the method of Lowry et al. [24]. Except for the chromatographic experiments, the enzyme activity values presented are the mean values of assays conducted in triplicate.

2.4. Purification of α-galactosidase

2.4.1. Step-1: Acetone precipitation

The pre-cooled acetone was added slowly to crude enzyme extract with constant stirring to give a concentration of 150% (v/v), and this solution was left on ice for 10 min. The precipitate, collected after centrifugation (10,000 rpm) for 15 min at 4 °C, was dissolved in sodium phosphate buffer (10 mM, pH 7.0), dialyzed against the same buffer and then used for further purification.

2.4.2. Step-2: DEAE-Sephadex chromatography

The enzyme preparation was loaded onto a DEAE-Sephadex A-50 column (30 cm × 1.5 cm) pre-equilibrated with 10 mM sodium phosphate buffer, pH 7.0. After loading of the partially purified enzyme, the unbound fractions were collected and checked for α-galactosidase activity, and then the column was washed with the equilibration buffer. The enzyme was eluted with 50 mM sodium phosphate buffer, pH 7.0. Fractions of 5 ml were collected at a flow rate of ~15 ml/h. The single activity peak fractions were pooled, concentrated and stored at 4 °C for further purification.

2.4.3. Step-3: Sephadex G-100 chromatography

The enzyme solution obtained from step 2 was loaded onto a Sephadex G-100 column (50 cm × 1.5 cm) pre-equilibrated with 100 mM sodium phosphate buffer, pH 7.0. The protein was eluted with the same buffer at a rate of ~10 ml/h. The single activity peak fractions were pooled, concentrated and dialyzed against the same buffer. This dialyzed enzyme solution was then used as purified enzyme preparation.

2.5. Polyacrylamide gel electrophoresis

The purity of the enzyme was checked using 10% denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The polyacrylamide gel was prepared by the method of Laemmli [25]. A wide range molecular mass calibration mixture (Sigma–Aldrich, USA) was used as the standard protein marker. The gel was stained with Coomassie Brilliant Blue R-250.

Native PAGE was performed using 10% polyacrylamide gel according to the method of Walker [26]. The protein bands in the gel were stained with Coomassie Brilliant Blue R-250. α-Galactosidase activity in the non-denaturing polyacrylamide gel was assayed by incubating the gel at 50 °C for 15 min in McIlvaine buffer (pH 5.0) containing the fluorescent substrate 4-methyl-umbelliferyl-α-D-galactopyranoside (2.0 mM). The activity band was visualized as a fluorescent band under UV light at 365 nm.

2.6. Optimum pH and stability

The optimum pH of α-galactosidase was determined by assaying α-galactosidase activity using a standard assay method over a pH range of 3.0–9.0 using either 100 mM citrate phosphate buffer (pH 3.0–7.5) or potassium phosphate buffer (pH 8.0–9.0). Enzymatic activities at different pH were expressed as relative values (%) with reference to 100% activity at the optimal pH.

The pH stability was determined by pre-incubating the enzyme in 100 mM citrate phosphate buffer (pH 3.0, 4.0, 5.0, 6.0 and 7.0), potassium phosphate buffer (pH 8.0 and 9.0) and glycine–NaOH buffer (pH 10.0) for a period of 3 h at room temperature. Small aliquots (50 μl) were withdrawn from all the samples, and residual enzyme activity was determined by standard assay methods.

2.7. Optimum temperature and stability

The optimum temperature of α-galactosidase was determined by performing assays at temperatures ranging from 20 °C to 100 °C. The thermal stability of the enzyme was determined by incubating enzyme at temperatures ranging from 50 °C to 80 °C for several time periods. The residual activity of the enzyme was estimated under standard assay conditions in each case and expressed as relative activity (%) with reference to the activity observed before incubation.

2.8. Effect of metal ions and reagents on α-galactosidase 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 room temperature, 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.

2.9. Effect of substrate concentration

The effect of varying the substrate concentration on the reaction rate was studied using o-nitrophenyl-α-D-galactopyranoside, melibiose and raffinose. The apparent Michaelis constant (K_m) for each substrate was determined using the method described by Lineweaver and Burk [27].

2.10. Substrate specificity

The substrate specificity of α-galactosidase towards various synthetic (oNPG, p-nitrophenyl-β-D-galactopyranoside and o-nitrophenyl-β-D-galactopyranoside) and natural glycosides (melibiose and raffinose) was determined. Substrates were prepared in McIlvaine buffer (pH 5.0) and were used for the enzyme assay using standard assay conditions with 50 μl of purified α-galactosidase. Data were expressed as k_{cat} values obtained with different substrates.

2.11. Transglycosylation activity of α-galactosidase

Transglycosylation was carried out in a reaction mixture containing 200 mM sodium phosphate buffer (pH 5.0) in the presence of 1 unit of α-galactosidase per ml, 50 mM o-nitrophenyl-α-D-galactopyranoside as a galactosyl donor, and 100 mM glucose as an acceptor. The incubation was done at 50 °C for 18 h, and the reaction was terminated by heating in a boiling water bath for 5 min. The reaction products were detected by thin layer chromatography (TLC) on silica-gel-coated glass plates.

2.12. TLC analysis

The products of transglycosylation reactions were analyzed by TLC (pre-coated silica-gel glass plates). Plates were developed at room temperature in a saturated chamber containing n-propanol and water (8:2, v/v) as a solvent system. After development, the TLC plates were sprayed with 0.1% orcinol dissolved in 5% H_2SO_4. The plates were then kept in an oven at 140 °C for 15 min for visualization of sugar spots.