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Short communication

Production, purification and partial characterization of a novel endo-β-1,3-glucanase from *Agaricus brasiliensis*

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Abstract

An endo-β-1,3-glucanase from *Agaricus brasiliensis* ATCC 76739 was first produced and purified in the submerged culture. Purified endo-β-1,3-glucanase with a 13.1-fold purification and 6.7% yield was prepared by two steps: precipitation with 70% saturation ammonium sulfate and chromatography on hydrophobic interaction chromatography. The molecular mass of the enzyme was estimated to be 33 kDa by SDS-PAGE. The presence of endo-β-1,3-glucanase might explain the fall of the yield of bioactive exopolysaccharides in the late stage of the submerged cultures. The pH optimum for the enzyme was 4.5, and the temperature optimum was 45 °C. The enzyme showed high pH stability within the range of pH 3.5–6.0 and thermostability up to 50 °C, and exhibited a half-life of 30 min at 55 °C, which was better than that of a thermophilic fungus, *Scytalidium thermophilum*. The enzyme activity was strongly inhibited by $HgCl_2$.

Keywords: *Agaricus brasiliensis*; Endo-β-1,3-glucanase; Purification; Exopolysaccharides; Thermostable; Characterization

1. Introduction

β-Glucans are not only the main structural components of the cell walls of most fungi but also responsible for the antitumor bioactivity [1–3] and the anti-diabetic activity [4] in several animal studies. The production of polysaccharides by the edible and medicinal mushroom *Agaricus brasiliensis* (previously named *Agaricus blazei*) has attracted considerable interest due to their strong antimutagenic effect [5,6], and information is available on the culture conditions which are known to affect their production [7,8]. Many studies indicated that the polysaccharides of *A. brasiliensis* are heteropolysaccharides [9,10]. The β(1 → 3) backbone and the β(1 → 6) branch of polysaccharides are probably responsible for their anti-tumor activity [11–13].

Edible mushrooms such as *Agaricus bisporus*, *Lentinula edodes* and *Pleurotus sajor-caju* produce endo-β-1,4-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) when cultivated on glucan and cellulose rich materials in either submerged culture or in solid-state systems [14,15]. However, most studies on the production and regulation of fungal endoglucanases focused on *Trichoderma reesei* [16–18] and *Phanerochaete chrysosporium* [19,20], and studies of endoglucanases of edible mushrooms except *A. bisporus* are limited [14]. Fungal glucanases were commonly responsible for the degradation of exopolysaccharides such as pullulans in the late stage of the submerged cultures [21–23].

Enzymatically hydrolyzed oligosaccharides from *A. brasiliensis* by a bacterial glucanase have been shown to have twice the anti-diabetic activity in diabetic rats [4]. Thus, characterization of the glucanase of this economically important edible mushroom *A. brasiliensis* would be essential for a better understanding of its biological efficiency (i.e. conversion of growth substrate into mushroom fruit bodies) in the solid-state systems and the production of the bioactive polysaccharides and oligosaccharides. In this study, we first report the purification and characterization of an extracellular endo-β-1,3-glucanase produced by *A. brasiliensis* when grown in an air-lift bioreactor.

2. Materials and methods

2.1. Microorganism and culture conditions

A. brasiliensis ATCC 76739 was grown in a culture medium containing (g/l): glucose 10; peptone 5; yeast extract 3; malt extract 3; KH_2PO_4 3; $MgSO_4{\cdot}7H_2O$ 0.3; Vitamin B_1 0.01 [8]. The pH of the medium was adjusted with 1 M HCl and

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0.5 M NaOH to 5.2. *A. brasiliensis* was cultivated at 28 °C with 0.2 vvm aeration rate in a 2 l air-lift bioreactor for 25 day. Cell mass in the broth was removed by filtration through Whatman paper No. 1 in Millipore filtration apparatus, and the filtrate was extensively dialyzed against water at 4 °C.

2.2. Enzyme purification

The purification procedure for endo-β-1,3-glucanase from *A. brasiliensis* involves two steps.

2.2.1. Step 1: ammonium sulphate precipitation

Ammonium sulfate was added to the filtrate to give a concentration of 70% (w/v) saturation at 4 °C [14]. Precipitation was allowed for 10 h, and followed by centrifugation at 6000 rpm in a Hettich MIRO 22R refrigerated centrifuge for 20 min. The precipitate was dissolved in a minimal amount of 50 mM acetate buffer (pH 5) containing 1 mM EDTA, and dialyzed for 24 h with three changes in the same buffer.

2.2.2. Step 2: DEAE–Sepharose column chromatography

Crude enzyme was applied to a phenyl–Sepharose (Amersham Bioscience, Piscataway, NJ, USA) column (1.6 cm × 20 cm) previously equilibrated with the 50 mM acetate buffer (pH 5) containing 1.0 M ammonium sulfate. After washing with two bed volumes of the initial buffer, elution was performed with a linear gradient of 1.0–0.0 M ammonium sulfate at a flow rate of 60.0 ml/h. Fractions showing β-1,3-glucanase activity were pooled, concentrated by ultrafiltration (Centricon 10, Amicon Division, W.R. Grace & Co.) and stored at −20 °C.

2.3. β-1,3-Glucanase assay and protein determination

β-1,3-Glucanase activity was routinely assayed by incubating 5 mg laminarin (β-1,3-glucan, Sigma), in 50 mM potassium acetate buffer, pH 5.0, with 1 ml of enzyme solution appropriately diluted in the same buffer [24]. After 30 min of incubation at 50 °C, the reaction was stopped by heating at 100 °C for 10 min. Then, the reducing sugars contents were determined by using the 3,5-dinitrosalicylic acid (DNS) [25]. One unit of β-1,3-glucanase was defined as the amount of enzyme that catalyzes the release of 1 μmol of glucose equivalent per min.

Protein concentration was determined by the method of Bradford [26], with bovine serum albumin as the standard.

2.4. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in Laemmli's system [27], using 4% acrylamide in the stacking gel and 12.5% acrylamide in the separating gel. Protein bands were visualized by staining with Coomassie R 250 brilliant blue. Low-molecular-mass standard proteins (Amersham Biosciences) were used for molecular mass determination as follows: phosphorylase b (94 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

2.5. Substrate specificity

The activity of the purified β-1,3-glucanase was tested on various polymers with α- or β-glycosidic bonds at a final concentration of 5 mg/ml. In each case, degradation was assayed by the production of reducing sugars and measured as described above. Substrate blanks were included in parallel. Besides, a typical substrate for exo-β-glucanases *p*-nitrophenyl-β-D-glucopyranoside (pNPG) was included, and the enzyme activity toward pNPG was measured by the amount of *p*-nitrophenol liberated from pNPG at 410 nm as described elsewhere [28].

2.6. Determination of pH and temperature optimum and their stability

The pH optimum of the β-1,3-glucanase was determined by performing the standard enzyme assay except the appropriate buffers containing 100 mM each component within the pH range of 3.0–8.0: sodium acetate (pH 3.0–5.0), phosphate (pH 5.0–7.0), and Tris–HCl (pH 7.0–8.0). The temperature optimum of the β-1,3-glucanase was determined by performing the standard assay within the temperature range of 25–65 °C. The thermal stability of the purified glucanase was examined by measuring the residual activity after incubation the enzyme mixture at each desired temperature for 30 min.

2.7. Determination of the effect of metal ions

The effects of several metal ions and compounds on β-1,3-glucanase activity were investigated. The purified enzyme (12.5 U/mg) was preincubated with each 1 mM metals or organic compounds for 15 min at 50 °C. The β-1,3-glucanase activity was determined by the standard assay as described above using laminarin as substrate. 100% activity corresponds to no reagents added.

2.8. Pattern of enzymatic hydrolysis

Products released following time-course incubation (0–2 h) of laminarin solution with the purified enzyme, at 50 °C and pH 5.0, were separated and analyzed using NH_2-HPLC (Nucleosil 10 NH_2, 4.6 × 250 mm, GL Science, Japan) with RI detector (SFD, RI2000) [29]. The mobile phase was water–acetonitrile mixture (2:8) with a flow rate of 1.0 ml/min.

3. Results and discussion

3.1. Enzyme production

Enzyme production was accomplished by using a glucan containing complex medium in an air-lift bioreactor. The activity of the endo-β-1,3-glucanase in the broth increased with mycelial biomass, but the activity reached its maximum, 2.5 U/ml, after 18 days of cultivation in the stationary phase as shown in Fig. 1. The presence of endo-β-1,3-glucanase activity might partially contribute to the fall of the yield of bioactive exopolysaccharides in the late stage of the submerged cultures [8].

3.2. Purification of glucanase and size determination

Purification of an endo-β-1,3-glucanase produced by *A. brasiliensis* was accomplished by a two-step procedure: precipitation with 70% saturation of ammonium sulfate and hydrophobic interaction chromatography on phenyl–Sepharose. Chromatography on phenyl–Sepharose resulted in the

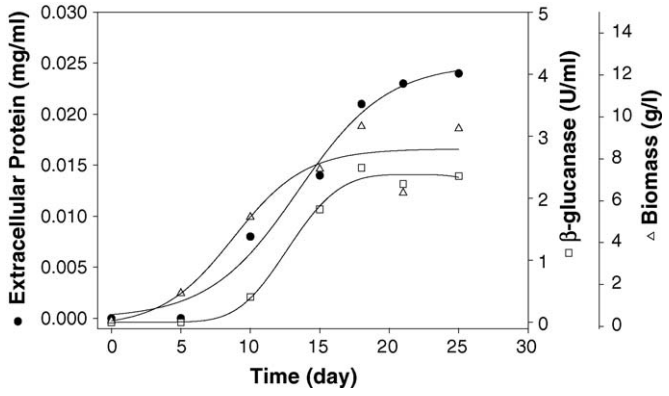

Fig. 1. Extracellular production of endo-β-1,3-glucanase from *A. brasiliensis* in air-lift bioreactor during growth in 1.0% glucose supplemented culture: (●) extracellular protein (mg/ml); (△) biomass (g/l); (□) β-1,3-glucanase activity (U/ml).