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A highly thermostable β-glucosidase activity from the thermophilic fungus *Humicola grisea* var. *thermoidea*: purification and biochemical characterization

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Abstract

Humicola grisea var. *thermoidea* grown in sugar cane bagasse produced and secreted two major protein components exhibiting β-D-glycosidase activities (forms I and II). Form I was purified to apparent homogeneity (PAGE and SDS-PAGE) by a three-step procedure involving acetone precipitation, DEAE-cellulose chromatography and filtration in Bio-Gel P-100. The purified enzyme was a glycoprotein of 35% carbohydrate content and apparent molecular mass of 55 kDa (SDS-PAGE and gel filtration), Optima of temperature and pH were 50–60°C and 6.0, respectively. The enzyme activity was stable at 60°C and exhibited a half-life of 30 min at 70°C. The enzyme hydrolyzed *p*-nitrophenyl β-D-glucopyranoside, cellobiose, xylobiose, *p*-nitrophenyl β-D-xylopyranoside and CM-cellulose. Kinetic studies indicated that these substrates were hydrolyzed at the same catalytic site.

Keywords: β-Glucosidase; β-Xylosidase; Cellobiase; Xylosidase; CM-case; *Humicola grisea*

1. Introduction

The existence of different of glycosidases with wide stereospecificity at the C-4 position of the pyranose ring has been described in several organisms [1–5]. These enzymes are classified as β-D-glucosidases, β-D-galactosidases, or β-D-fucosidases, considering their relative efficiency of substrate hydrolysis (V_{max}/K_m ratio). β-D-Glucosidases have been found in a variety of organisms including bacteria, yeasts, fungi, plants and animals. In cellulolytic microorganisms, the main role of β-D-glucosidase activity is the hydrolysis of cellobiose and short chain cellooligosaccharides, producing glucose.

The thermophilic fungus *Humicola grisea* var. *thermoidea* is known as a good source of β-D-glucosidase activity [5,6]. Previous work from our laboratory reported the purification and biochemical characterization of a multifunctional intracellular β-D-glucosidase activity from this organism [5]. The present study describes the purification and some

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biochemical properties of an extracellular β-D-glucosidase secreted by *H. grisea* var. *thermoidea*, which exhibits remarkable thermostability. This extracellular enzyme exhibited several differences, as compared with that purified from mycelial extracts [5].

2. Materials and methods

2.1. Organism and culture conditions

The *H. grisea* var. *thermoidea* strain was maintained on slants of solid 4% oat-meal baby food (Quaker) medium. For enzyme induction the fungus was grown as recommended by Peralta et al. [5] except that 1.0% sugar cane bagasse was used as the main carbon source. Liquid cultures were incubated at 45°C with shaking (145 rpm). After 20 h the cultures were harvested by filtration and the filtrate was saved as the crude preparation of β-D-glucosidase.

2.2. Enzyme assays

β-D-Glucosidase was determined at 50°C with 5.0 mM *p*-nitrophenyl-β-D-glucopyranoside (PNP-Glc) as substrate, in 50 mM potassium phosphate buffer (pH 6.0). After 10–20 min of incubation the reaction was stopped by adding two volume of a saturated solution of sodium tetraborate and the absorbance was read at 405 nm. β-D-Xylosidase was assayed with *p*-nitrophenyl-β-D-xylopyranoside (PNP-Xyl) as substrate under the conditions described above. Hydrolysis of cellobiose was measured in a reaction mixture containing 5 mM of substrate in 50 mM sodium phosphate buffer (pH 6.0). After 10–20 min at 50°C glucose released was estimated by the glucose oxidase method [7]. CM-case activity was assayed using 1% (w/v) CM-cellulose (Sigma; Na salt; medium viscosity), as described above. Reducing sugar was estimated by the dinitrosalicylic acid method [8]. Enzyme units were defined as the amount that produced 1 μmol of *p*-nitrophenol (β-D-glucosidase and β-D-xylosidase) or glucose (cellobiase and CM-case) per min. Visualization of enzymatic activity in PAGE was performed as described in Peralta et al. [5], using 6-bromo-2-naphthyl β-D-glucopyranoside as substrate

2.3. Purification of β-D-glucosidase

Acetone at −20°C was added to the culture filtrate to achieve 75% (v/v) final concentration. After 1 h in an ice bath, the precipitate was collected by centrifugation (10 000 × *g*; 30 min), dissolved in a small volume of 50 mM potassium phosphate buffer (pH 6.0) (phosphate buffer) and dialyzed overnight against the same buffer. Undissolved material was removed by centrifugation and the supernatant was applied to a DEAE-cellulose column (1.4 × 24.0 cm) equilibrated with phosphate buffer and eluted with a linear gradient (0–0.3 M) of sodium chloride dissolved in the same buffer. β-D-Glucosidase was evenly distributed into two activity peaks (forms I and II). Form I did not bind to the resin while form II was eluted with 0.16 M salt (data not shown). Form I was precipitated with acetone, collected by centrifugation, dissolved in a small volume of 100 mM potassium phosphate (pH 6.0) containing 100 mM KCl and applied on a Bio-Gel P-100 column (1.5 × 45.0) equilibrated and eluted with the same buffer. Fractions with β-glucosidase activity were pooled, dialyzed against water and lyophilized.

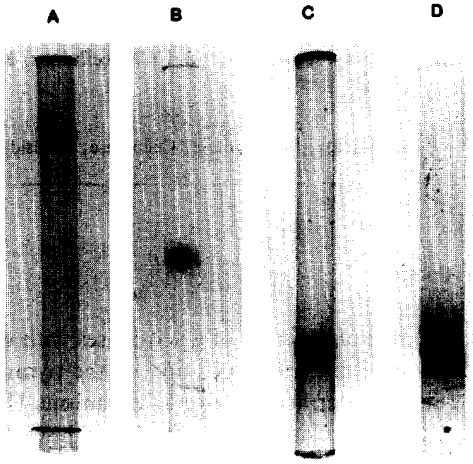

Fig. 1. Polyacrylamide (7%) gel electrophoresis of the purified β-glucosidase. (A) PAGE; (B) SDS-PAGE; (C) activity test for aryl-β-D-glucosidase with 6-bromo-2-naphthyl β-D-glucopyranoside; (D) PAGE. A and B samples not treated with *n*-hexane; C and D received treatment with *n*-hexane. A, B and D were stained with Coomassie blue.

2.4. Analytical methods

Protein was assayed according to the method of Lowry et al. [9] using bovine serum albumin as a standard. Total neutral carbohydrate was estimated using the method of Dubois et al. [10]. Hydrolysis products were analyzed by thin-layer chromatography on silica gel G-60 plates.

3. Results and discussion

3.1. Production of extracellular β-D-glucosidase activity

H. grisea secreted high levels of β-D-glucosidase activity in culture media supplemented with cellulosic materials as the main source of carbon. Total enzyme units in filtrates of cultures supplemented with 15% (w/v) of either Avicel, CM-cellulose, corn cob, or sugar cane bagasse were almost the same, but the specific activity was 4-fold greater in the presence of sugar cane bagasse. β-D-Glucosidase activity was about 60-fold lower for cultures grown in glucose or in a mixture of glucose and cellulosic materials, suggesting that the enzyme was affected by carbon catabolic repression.

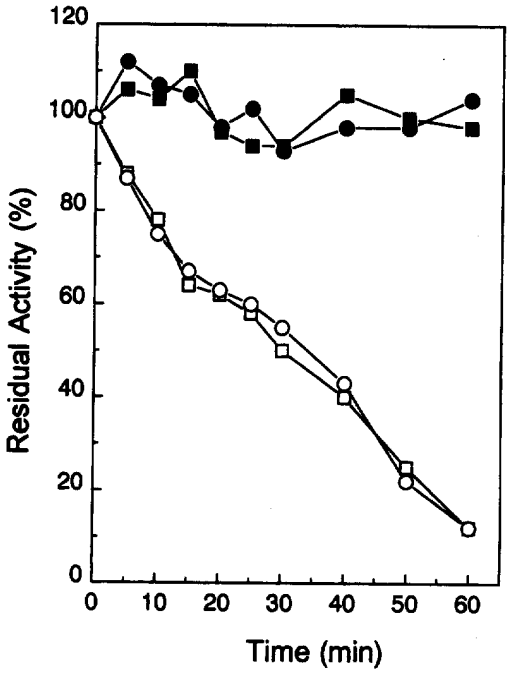

Fig. 2. Thermal inactivation at 60°C (closed symbols) and 70°C (open symbols) of β-D-glucosidase (●,○) and β-D-xylosidase (■,□) activities.

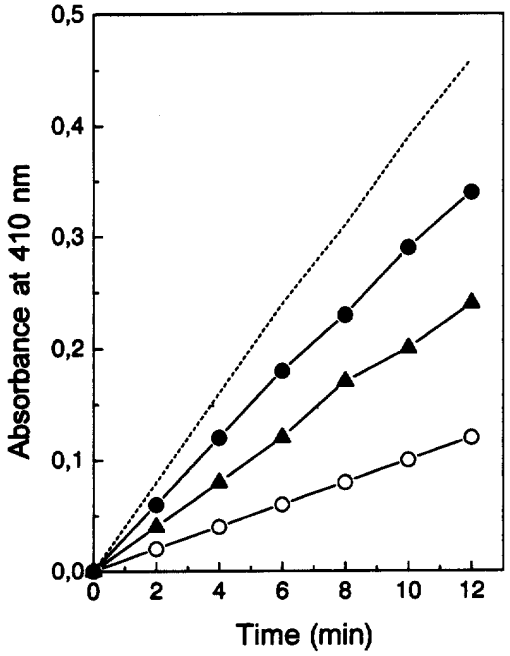

Fig. 3. Hydrolysis of PNP-Glc (•), PNP-Xyl (o) and mixtures of the two substrates (▲) as a function of time. (- - -) Theoretical rate of hydrolysis of a mixture of the two substrates assuming separate enzymes.

3.2. Properties of the purified β-D-glucosidase

After Bio-Gel P-100 filtration, β-D-glucosidase (form I) specific activity was about 2.7 ± 0.4 U (mg protein)$^{-1}$ ($n = 15$), and 38-fold purification was achieved. The preparation gave a single band in either PAGE or SDS-PAGE (Fig. 1A,B). Electrophoretic (PAGE) migration was improved and the protein band appeared sharper, for samples extracted three times with an excess (15-fold) of *n*-hexane (Fig. 1C,D), suggesting that this treatment removed lipids associated with the purified protein.

The carbohydrate content of the purified enzyme was estimated to be 35%, a value higher than that reported for the intracellular β-D-glucosidase purified from *H. grisea* [5].

Molecular sieving (Sephadex G-100) and SDS-PAGE indicated a molecular mass of 55 kDa, suggesting that the purified β-D-glucosidase was a monomeric protein. This value was comparable to that obtained for the intracellular β-glucosidase purified from *H. grisea* [5].

3.3. Effects of temperature and pH on activity and stability

Maximum β-D-glucosidase activity was achieved at 50°C which remained constant up to 60°C, attaining 88% of its maximum at 80°C. The enzyme was more active at pH 6.0, and was stable up to 60 min at 50°C over a pH range of 4.0–8.0.

3.4. Effects of metal ions

Added at 1.0 mM concentration Mg^{2+}, Ca^{2+}, Al^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+} and Mn^{2+} had no effect on β-D-glucosidase activity, while Hg^{2+} and Ag^{2+} practically inhibited all activity

3.5. Kinetic parameters

Apparent K_m and V_{max} were calculated from Lineweaver-Burk plots. K_m values ($n=8$; expressed in mM) were 0.12 ± 0.01, for PNP-Glc, 3.24 ± 0.3 for cellobiose and 0.44 ± 0.03 for PNP-Xyl. The efficiency of substrate utilization was estimated on the basis of V_{max}/K_m ratios. We found those ratios to be 27 for PNP-Glc, 0.91 for PNP-Xyl, and 0.26 for cellobiose, indicating that the best substrate was PNP-Glc. The K_m value of the polymeric substrate CM-cellulose was 4.57 ± 1.05 $(mg/ml)^{-1}$.

3.6. Thermal inactivation of purified β-glucosidase

Thermal inactivation kinetics were determined by incubating the purified enzyme dissolved in water at 60 and 70°C. Subsequently, the heated samples were quickly chilled in an ice bath and assayed with PNP-Glc or PNP-Xyl as substrates under the conditions described above. The enzyme activity was fully stable up to 60 min at 60°C, and exhibited a half-life of about 30 min at 70°C (Fig. 2). These data indicated that the extracellular β-glucosidase was more resistant to temperature than the intracellular enzyme [5], which exhibited a half-life of about 12 min at 55°C.

3.7. Evidence for the hydrolysis of PNP-Glc, PNP-Xyl, CM-cellulose and cellobiose at a common catalytic site

The possibility of a common catalytic site for hydrolysis of the synthetic substrates PNP-Glc and PNP-Xyl was examined by the mixed substrate kinetic method [12]. The rate of hydrolysis of the substrate mixture was not additive relative to the rates obtained when each substrate was tested separately (Fig. 3). The presence of CM-cellulose or cellobiose inhibited competitively the hydrolysis of PNP-Glc or PNP-Xyl with K_i of 3.9 $g.l^{-1}$ and 0.45 mM for CM-cellulose and cellobiose, respectively. These results indicated the existence of a unique catalytic site for hydrolysis of all substrates found for the enzyme.

3.8. Enzyme specificity

The purified β-D-glucosidase hydrolyzed PNP-Glc (100%), cellobiose (20%), CM-cellulose (8%) and PNP-Xyl (10%). *o*-Nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl β-D-fucopyranoside, *p*-nitrophenyl α-D-glucopyranoside, lactose, Avicel, xylan and starch did not serve as substrates. The mechanism of action of the purified enzyme on CM-cellulose was analyzed by thin-layer chromatography. Glucose was the only product detected even after a 12 h incubation, suggesting that the enzyme acted as an exocellulase. The purified enzyme also hydrolyzed cellotriose and xylobiose. According to substrate specificity, the extracellular β-glucosidase of *H. grisea* behaved differently from the mycelial enzyme, which was not able to hydrolyze CM-cellulose, but did hydrolyze *o*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-fucopyranoside and lactose [5]. Furthermore, the β-D-xylosidase purified previously from *H. grisea* [11] was unable to hydrolyze CM-cellulose and PNP-Glc. Thus, the combined action of the two different β-glucosidase activities present in *H. grisea* could improve the capacity of the organism to utilize cellobiose and xylobiose released from organic materials by the action of the enzymes of the cellulolytic and hemycellulolytic complexes.

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