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Purification and characterization of the intracellular β-glucosidase from *Aureobasidium* sp ATCC 20524

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β-Glucosidase hydrolyzing cellobiose was extracted from *Aureobasidium* sp ATCC 20524 and purified to homogeneity. The molecular mass was estimated to be about 331 kDa. The enzyme contained 26.5% (w/w) carbohydrate. The optimum pH and temperature for the enzyme reaction were pH 4 and 80°C, respectively. The enzyme was stable at a wide range of pH, 2.2–9.8, after 3 h and at 75°C for 15 min. The kinetic parameters were determined. The enzyme was relatively stable against typical organic enzyme inhibitors. The enzyme also hydrolyzed gentiobiose, *p*-nitrophenyl-β-glucoside and salicin.

Keywords: β-glucosidase; cellobiase; cellobiose-hydrolysis; *Aureobasidium*

Introduction

β-glucosidase (β-d-glucoside glucohydrolase, EC 3.2.1.21), which catalyses the hydrolysis of β-glucosidic linkages, has been reported from many kinds of microorganisms [18,22]. Cellobiase plays an important role in the development of processes for the saccharification of cellulose to obtain fermentable sugars [2,14].

Thermostable β-glucosidases [19,22] and acid stable ones [11,16] have been reported. We reported the production of stable intracellular β-glucosidase by *Aureobasidium* sp and noted its high pH and thermal stability [9]. Subsequently, the detailed properties of extracellular β-glucosidase from *Aureobasidium pullulans* using *p*-nitrophenyl-β-d-glucopyranoside as substrate was reported [15].

In the present paper, we describe the purification and cellobiose-hydrolyzing characteristics of a stable intracellular β-glucosidase from *Aureobasidium* sp ATCC 20524 and compare them to those of the cellobiase activities from other sources.

Materials and methods

Microorganism

Aureobasidium sp ATCC 20524 which was maintained on agar slants (sucrose 1%, yeast extract 0.2%, agar 1.8% and pH 6.5–7) was used.

Cultivation and preparation of crude enzyme

Cultivation of the strain for enzyme production was carried out in liquid culture (lactose 15%, yeast extract 2.0%, K_2HPO_4 0.2%, $MgSO_4$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.03%, pH 7) at 30°C for 48 h with the same conditions described in a previous report [9]. Culture broth was centrifuged and the cells were harvested. The enzyme was solubilized from cells by 0.1% benzalkonium chloride in 75 mM McIlvain (citric acid/Na_2HPO_4) buffer at 30°C for 24 h and ultrasonic disintegration at 20 kc for 2 min on ice. After centrifugation, the resultant supernatant was used for enzyme purification.

Purification of the enzyme

The crude extract was passed through a DEAE-Toyopearl 650S anion exchange column (1.0 × 40 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8) and dialyzed against 20 mM acetate buffer (pH 4). The dialyzate was applied on a S-Sepharose Fast Flow cation exchange column (1.0 × 40 cm) equilibrated with 20 mM phosphate buffer (pH 4). The column was washed with 20 mM phosphate buffer (pH 4) and then eluted with the same buffer containing 0–1 M NaCl. The enzyme fractions were collected and concentrated with a membrane filter (Amicon PM 10; Amicon, Tokyo, Japan). The enzyme solution from the previous stage was applied on a TSK-Gel Toyopearl HW55F gel filtration column (1.5 × 100 cm) equilibrated with 20 mM acetate buffer (pH 5) containing 0.2 M NaCl. After application of the enzyme to the column, it was eluted with the same buffer at a flow rate of 10 ml h^{-1}. The chromatographic steps were carried out at 20°C.

Molecular mass estimation

The molecular mass of the enzyme was estimated by TSK-Gel Toyopearl HW55F (1.5 × 100 cm) gel filtration according to the method of Andrews [1]. The column was equilibrated with 20 mM acetate buffer (pH 5) containing 0.2 M NaCl. After application of the purified enzyme on the column, it was eluted with the same buffer at a flow rate of 10 ml h^{-1}. The elution of protein standards (ferritin, 450 kDa; catalase, 240 kDa; aldolase, 158 kDa and bovine serum albumin, 68 kDa; Boehringer-Mannheim, Tokyo, Japan) was carried out in the same manner.

Electrophoresis

Disc-electrophoresis of the purified enzyme was performed at a constant of 3 mA per gel on a 7.5% (w/w) polyacrylamide gel at pH 8.3 according to the method of Davis [5]. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE) was carried out in 7.5% (w/v) acryl-

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Table 1 Purification of β-glucosidase from *Aureobasidium* sp ATCC 20524

Step	Activity (U)	Protein (mg)	Specific activity (U mg^{-1} protein)	Yield (%)
Crude extract	377	894	0.422	100
DEAE-Toyopearl 650S	347	670	0.518	92.0
S-Sepharose Fast Flow	188	12.5	15.0	49.9
TSK-Gel Toyopearl HW55F	109	4.80	22.7	28.9

amide and 0.1% (w/v) SDS with a discontinuous Tris-glycine buffer system by the method of Laemmli [12]. The following proteins were used as molecular weight markers (Amersham Pharmacia Biotech, Tokyo, Japan): myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa) and glutamic dehydrogenase (53 kDa). After electrophoresis, the gel was stained with Coomassie blue.

Assays

Enzyme activity was determined in 50 mM McIlvain buffer (pH 4) which contained a specific amount of enzyme and 50 mM cellobiose as substrate. The reaction mixture was incubated at 80°C for 20 min.

Glucose released in the reaction mixture was assayed by the glucose oxidase method (Glucose test B; Wako, Osaka, Japan). One unit of enzymatic activity was defined as the quantity of enzyme required to produce 2 μmol of glucose per min from cellobiose or 1 μmol of glucose per min from other substrates.

Protein was assayed by the method of Lowry *et al* [13] with bovine serum albumin (Nakalai, Kyoto, Japan) as standard. Carbohydrate were assayed by the method of Dubois *et al* [7] with glucose (Wako, Osaka, Japan) as standard.

Results and discussion

Purification of the enzyme

The results of purification of β-glucosidase from *Aureobasidium* sp ATCC 20524 are shown in Table 1. The specific activity of the final purified preparation was 22.7 U mg^{-1} protein representing a purification factor of 53.8. The enzyme showed a single band by Disc-PAGE (Figure 1). The specific activity of the present enzyme was similar to that (25.3 U mg^{-1} protein) of the enzyme from *Hansenia vineae* using cellobiose as substrate for enzyme assay [21].

Molecular mass of the enzyme, determined by gel filtration using TSK-Gel Toyopearl HW55F, was estimated to be about 331 kDa and the carbohydrate content was 26.5% (w/w). The molecular mass of the enzyme is similar to that of the enzyme from *Hanseniaspora* sp (295 kDa) [21], *Aureobasidium pullulans* (340 kDa) [15] and *Aspergillus niger* (360 kDa) [23]. The carbohydrate content of the enzyme was similar to that (30.5%) of the enzyme from *Candida wickerhamii* [8]. The subunit (57 kDa) of the enzyme was detected in SDS-PAGE. The molecular mass was similar to that of the *Pyrococcus furiosus* enzyme (58 kDa) [10].

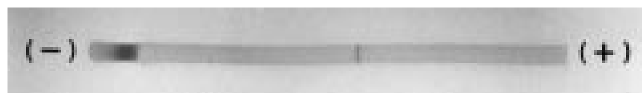

Figure 1 Disc-PAGE of the purified β-glucosidase from *Aureobasidium* sp ATCC 20524.

Effect of pH and temperature on the enzyme

The effect of pH on the activity and stability of the enzyme was investigated (Figure 2). The enzyme showed the highest activity at pH 4, similar to the enzyme from *Gliocladium virens* (pH 4.5) [20]. The enzyme was stable over a wide range of pH, 2.2–9.8, retaining more than 93% of its original activity after 3 h within this range.

The effect of temperature on the activity and stability of the enzyme was investigated (Figure 3). The optimum temperature was 80°C. The enzyme was stable at 75°C for 15 min, retaining 98% of its original activity and also remained 88% active at 80°C.

While the enzymes from *Aspergillus aculeatus* hydrolyzing salicin were not tested using cellobiose as substrate, they have high stabilities at acidic pH but are not stable at high temperature (<60°C) [16]. It is considered that the reaction at acidic pH and high temperature is favorable so as to limit contamination.

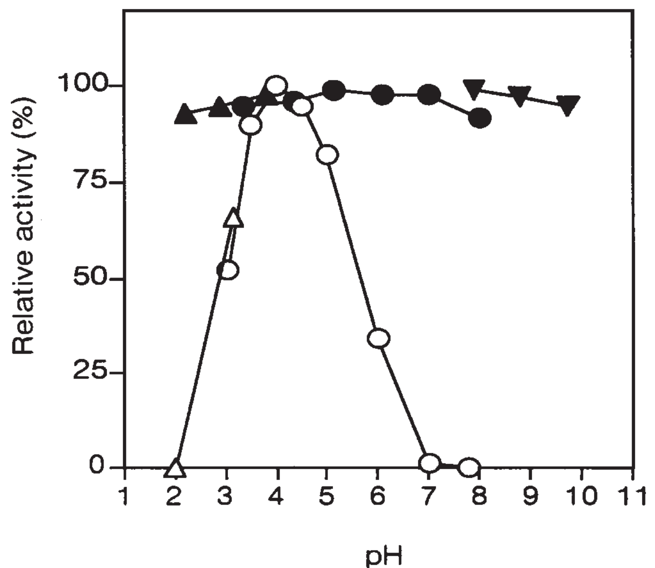

Figure 2 The effect of pH on the activity (open symbols) and stability (closed symbols) of β-glucosidase from *Aureobasidium* sp. The activities were measured after 3 h incubation at each pH and 25°C in citrate buffer (acidic pH, ▲), McIlvain buffer (acid to neutral pH, ●) and Michaelis buffer (NH_4Cl/NH_4OH, alkaline pH, ▼) according to the method described in Materials and Methods.

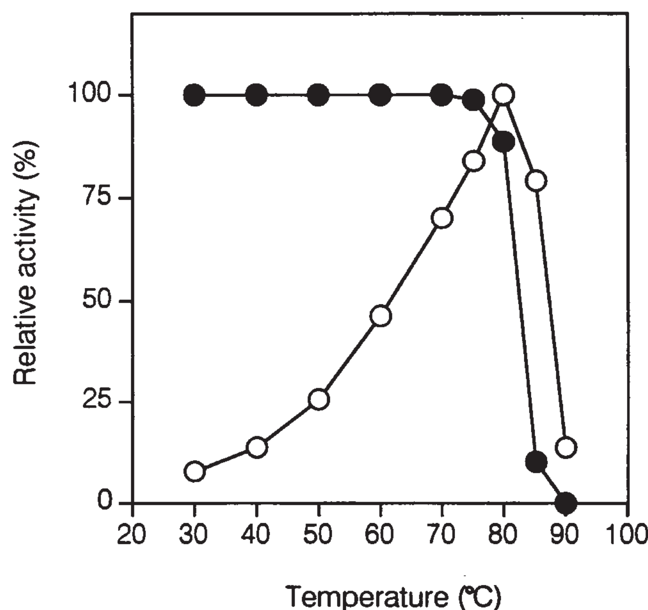

Figure 3 The effect of temperature on the activity (open circle) and stability (closed circle) of β-glucosidase from *Aureobasidium* sp. The activities were measured after 15 min incubation at each temperature and pH 5 as described in Materials and Methods.

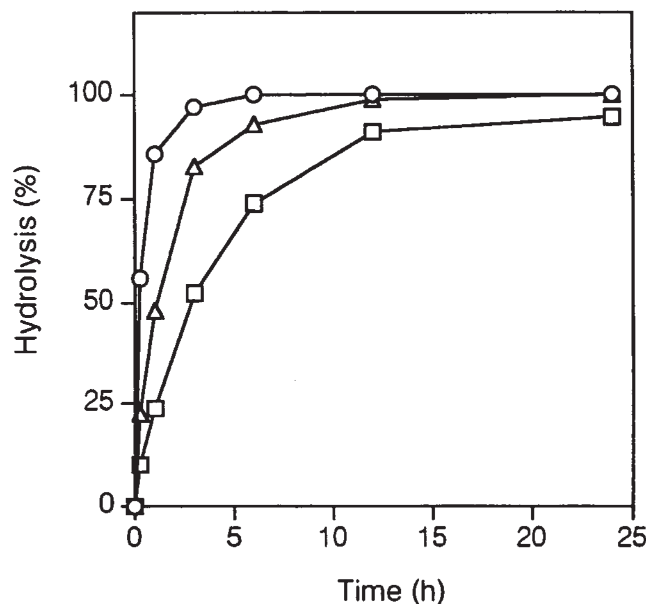

Figure 4 The time course of cellobiose hydrolysis by β-glucosidase from *Aureobasidium* sp. The reaction mixture described in Materials and Methods containing 0.26 U enzyme and cellobiose of each concentration (-○- 10 mM; -△- 25 mM; -□- 50 mM) was incubated at pH 4 and 80°C, and the amount of glucose released was measured at each time interval.

Kinetic parameter of the enzyme

The Michaelis constant (K_m) and maximum velocity (V_{max}) toward cellobiose were determined from a Lineweaver–Burk plot. The K_m was found to be 8.85 mmol L^{-1} and the V_{max} was 37.5 μmol min^{-1} mg^{-1} protein. From these data, molar activity (k_{cat}) and catalytic efficiency (k_{cat} K_m^{-1}) were calculated to be 152 s^{-1} and 1.72×10^4 s^{-1} M^{-1}, respectively.

The wide range of K_m and V_{max} values of microbial β-glucosidase toward cellobiose have been reported by many investigators. The values of the present enzyme were similar to those of the enzyme from *Aspergillus niger* (K_m, 5.63 mmol L^{-1}; V_{max} 33.74 μmol min^{-1} mg^{-1} protein) [6].

Time course of cellobiose hydrolysis

The time course of cellobiose hydrolysis by the enzyme is shown in Figure 4. Cellobiose at 10 mM was hydrolyzed completely, as for the *Aspergillus aculeatus* enzyme [16] after a 6-h reaction time and glucose was detected as the only product in the reaction mixture.

Effects of various chemicals and metal ions on enzymatic stability

While the enzyme was inhibited slightly by reducing reagents such as 2-mercaptoethanol and hydrazine, but was not inhibited by other organic inhibitors such as sulfhydryl-reagents (*p*-chloromercuribenzoic acid, monoiodoacetic acid), chelating reagents (ethylendiaminetetraacetic acid, nitrilotriacetic acid) and detergents (sodium dodecylsulfate, benzalkonium chloride).

The enzyme was inhibited strongly by metal ions of transition elements such as copper and silver (group 11; 13.0 and 33.0% respectively of the original activity) and lead (group 14; 35.5%), and less by mercury (group 12; 72.5%), aluminum (group 13; 63.5%) and iron (group 8; 82.8%). These elements generally combine with the functional groups of amino acid residues such as cysteine, tyrosine, lysine and histidine. Inhibition by these metal ions on β-glucosidases from many sources such as *Aspergillus niger* [17] have been reported.

The cellobiase activity of *Trichosporon adeninovorans* [3] and *Aspergillus niger* [17] were enhanced by calcium and cobalt ions, respectively. However, no significant effect of these ions was observed on the present enzyme.

Substrate specificity of the enzyme

The enzyme hydrolyzed oligosaccharides such as gentiobiose (104% compared to cellobiose) and glucosides such as salicin (68.7%), methyl-β-glucoside (43.7%) and *p*-nitrophenyl-β-glucoside (182%). The enzyme, however, did not hydrolyze the glycoside linkages of polysaccharides. Hydrolysis of the α-linkage of oligosaccharides such as maltose, and glucosides such as *p*-nitrophenyl-α-glucoside was also tested but was not observed. It is considered that the enzyme catalyses the hydrolysis of β-1,4 and β-1,6 glycosidic linkages of oligosaccharides and β-linkages of glucosides.

The pattern of substrate specificity was somewhat similar to that of the *Hanseniaspora vineae* [21] or *Penicillium oxalicum* [4] enzymes but was very different from that of the *Aureobasidium pullulans* enzyme [15].

In the present paper, we investigated the cellobiose-hydrolyzing characteristics of an intracellular β-glucosidase from *Aureobasidium* sp because cellobiase activity is important for the utilization of the enzyme in cellulose saccharification processes.

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