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Purification and properties of the cell-associated β-xylosidase from *Aureobasidium*

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β-Xylosidase was extracted from *Aureobasidium* sp. ATCC 20524 and purified to homogeneity. The molecular mass was estimated at 411 kDa. The enzyme contained 15.3% (w/w) carbohydrate. The optimum pH and temperature were pH 3.5 and 80°C, respectively. The enzyme was stable at pH 3.5–9 after 3 h and at 80°C after 15 min. The Michaelis constant (K_m) and maximum velocity (V_{max}) toward *p*-nitrophenyl-β-D-xyloside were 2.0 mmol l^{-1} and 0.94 mmol min^{-1} mg^{-1} protein, respectively. The enzyme was inhibited strongly by mercury, lead, and copper ions. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 276–279.

Keywords: *Aureobasidium*; *p*-nitrophenyl-β-D-xyloside; β-xylosidase

Introduction

Xylan is the most abundant polysaccharide in wood and agricultural residues. Hydrolysis of this biomass occurs by the combined action of microbial enzymes, including xylanase and β-xylosidase [1]. These xylan-degrading enzymes are useful in the pulp and paper industry [6].

β-Xylosidase (EC 3.2.1.37) catalyses the hydrolysis of β-glycosidic linkages of xylo-oligosaccharides and xylosides and is very important for complete hydrolysis of xylan. The enzymes have been reported from many microorganisms, such as *Aspergillus*, *Chaetomium*, and *Clostridium* [18]. Some characteristics of the purified extracellular β-xylosidases from *Aureobasidium pullulans* CBS 58475 [7] and *Au. pullulans* NRRL Y-2311-1 [6,17] have been reported. Recently, we purified the cell-associated β-xylosidase from another strain of *Aureobasidium* and found that the characteristics are different from those of enzymes reported previously.

In the present paper, we describe the purification and enzymatic characteristics of cell-associated β-xylosidase from *Aureobasidium* sp. ATCC 20524 because of its superior properties such as high catalytic efficiency. We also compare these characteristics to those of the enzymes from other sources including other *Aureobasidium* isolates.

Materials and methods

Microorganism and growth

Aureobasidium sp. ATCC20524 was grown in 100 ml of medium which consisted of (%, w/v): oat spelt xylan, 1; yeast extract, 2; K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.05; $FeSO_4$, 0.03; pH 5 at 30°C for 72 h. The culture broth was then centrifuged and cells were harvested. The enzyme was solubilized from cells by 0.03% (w/v) Triton X-100 and ultrasonic disintegration. After centrifugation, the resultant supernatant was used for enzyme purification.

Purification of the enzyme

The crude extract was applied to a DEAE-Toyopearl 650S column (3×30 cm) equilibrated with 20 mmol l^{-1} phosphate buffer (pH 6). The column was washed with the same buffer and then eluted with the buffer containing 0–1.2 mol l^{-1} NaCl. The enzyme fractions were collected and dialyzed against 20 mmol l^{-1} acetate–HCl buffer (pH 3). The dialysate was applied to an S-Sepharose Fast Flow column (3×30 cm) equilibrated with 20 mmol l^{-1} acetate–HCl buffer (pH 3). The column was washed with 20 mmol l^{-1} acetate–HCl buffer (pH 3) and then eluted with the same buffer containing 0–1 mol l^{-1} NaCl. Enzyme fractions were collected and concentrated with a membrane filter (Amicon PM10). The enzyme solution from the previous stage was applied on a Sephadex G-200 gel filtration column (1.5×100 cm) equilibrated with 20 mmol l^{-1} phosphate buffer (pH 6) containing 0.1 mol l^{-1} NaCl. After application of the enzyme to the column, it was eluted with the same buffer at a flow rate of 36 ml h^{-1}.

Molecular mass estimation

The molecular mass of the enzyme was estimated by Sephadex G-200 (1.5×100 cm) gel filtration according to the method of Andrews [2]. The column was equilibrated with 20 mmol l^{-1} phosphate buffer (pH 6) containing 0.1 mol l^{-1} NaCl. After application of the purified enzyme to the column, it was eluted with the same buffer at a flow rate of 36 ml h^{-1}. The elution of protein standards (ferritin, 450 kDa; catalase, 240 kDa; aldolase, 158 kDa; ovalbumin, 45 kDa; Boehringer-Mannheim, Tokyo, Japan) was carried out in the same manner.

Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) was carried out in 7.5% (w/v) acrylamide and 0.1% (w/v) SDS with a discontinuous Tris–glycine buffer system by the method of Laemmli [13]. 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

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dehydrogenase (53 kDa). After electrophoresis, the gel was stained with Coomassie blue.

Assays

Enzyme activity was determined in 50 mmol l^{-1} acetate–HCl buffer (pH 3.5) which contained a specific amount of enzyme and 5 mmol l^{-1} *p*-nitrophenyl-β-D-xyloside (PNPX) as substrate. The reaction mixture was incubated at 80°C for 10 min. The optical density of librated *p*-nitrophenol was measured at 410 nm. One unit of enzymatic activity was defined as the quantity of enzyme required to produce 1 μmol of *p*-nitrophenol in 1 min.

Protein was assayed by the Lowry method [14] with bovine serum albumin (Nakalai, Kyoto, Japan) as standard.

Carbohydrate was assayed by the method of Dubois *et al.* [8] 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 626 U mg^{-1} protein representing a purification factor of 70. While a specific activity of *Aspergillus terreus* enzyme (4233 U mg^{-1} protein) [4] using PNPX as substrate has been reported, the value of the present enzyme was relatively high in comparison to enzymes from *Talaromyces emersonii* (747, 149, and 96 U mg^{-1} protein) [18], *Humicola grisea* (175 U mg^{-1} protein) [1], *As. nidulans* (107 U mg^{-1} protein) [11], *Termitomyces clypeatus* (94 U mg^{-1} protein) [3], *Bacillus* sp. (44.8 U mg^{-1} protein) [22], and *Trichoderma harzianum* (3.42 U mg^{-1} protein) [21].

The molecular mass of the enzyme, determined by gel filtration using Sephadex G-200, was estimated to be about 411 kDa (Figure 1A) and the carbohydrate content was 15.3% (w/w). The subunit (135 kDa) of the enzyme was detected in SDS-PAGE (Figure 1B).

The molecular mass of the enzyme (411 kDa) was higher than that reported for *Penicillium verruculosum* (200 kDa) [5], *Bacillus* sp. (180 kDa) [22], *As. nidulans* (180 kDa) [11], *Sclerotium rolfsii* (170 kDa) [12], and *As. terreus* (95 kDa) [4] and was different from that (224 [7] and 216 [6] kDa) of the extracellular enzymes from *Au. pullulans*. The molecular mass of subunit (135 kDa) was similar to the *Au. pullulans* enzyme (121 kDa) [7], the *As. awamori* enzyme (110 kDa) [10], and the *S. rolfsii* enzyme (180 kDa) [12].

From these results, the present native enzyme was estimated to be trimeric in nature and different from the extracellular *Au. pullulans* enzyme that is dimeric [7]. Monomeric (*T. emersii* [19] and *S. rolfsii* [12]), dimeric (*P. verruculosum* [5], *As. nidulans* [11], and *Ta. emersonii* [19]), and trimeric (*Bacillus* sp. [22]) β-xylosidases hydrolyzing PNPX have been reported.

Table 1 Purification of β-xylosidase from *Aureobasodium*

Step	Activity (U)	Protein (mg)	Specific activity (U mg^{-1} protein)	Yield
Crude extract	4340	480	9	100
DEAE-Toyopearl 650S	3119	27	116	72
S-Sepharose Fast Flow	2671	5.0	534	62
Sephadex G-200	1503	2.4	626	35

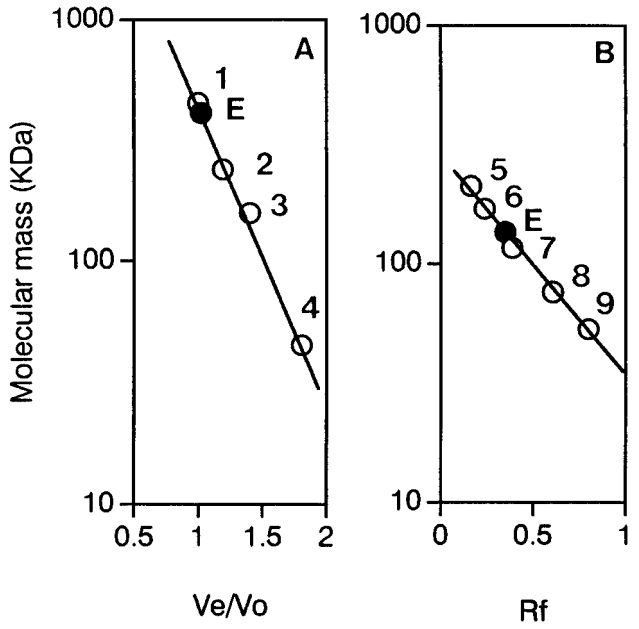

Figure 1 Estimation of the molecular mass of β-xylosidase from *Aureobasidium* by gel filtration and SDS-PAGE. (A) Logarithm of molecular mass of proteins against elution volumes from Sephadex G-200. E, the present enzyme. Standard proteins: (1) ferritin (450 kDa); (2) catalase (240 kDa); (3) aldolase (158 kDa); (4) ovalbumin (45 kDa). V_e and V_0 are the elution volume of the protein and void volume, respectively. (B) Plot of logarithm of molecular mass of proteins against R_f values in SDS-PAGE. E, the present enzyme. Standard proteins: (5) myosin (212 kDa); (6) α_2-macroglobulin (170 kDa); (7) β-galactosidase (116 kDa); (8) transferrin (76 kDa); (9) glutamic dehydrogenase (53 kDa).

The carbohydrate content of the present enzyme was higher than that of the enzymes from *Tr. virides* (4.5%) [15] and *Emericella nidulans* (4%) [16].

Effect of pH and temperature on the enzyme

The enzyme showed the highest activity at pH 3.5 (Figure 2), similar to the enzyme from *Ta. emersonii* (pH 3.5) [19] and relatively lower than that of enzymes from *P. verruculosum* (pH 4) [5], *Te. clypeatus* (pH 5) [3], and *As. awamori* (pH 6.5) [10]. The optimum pH of the present enzyme was different from that (pH 4.5) of the extracellular *Au. pullulans* enzyme [7]. The enzyme was stable over a wide range of pH (3.5–9), retaining more than 90% of its original activity after 3 h.

The optimum temperature was 80°C (Figure 3). This value was similar to that of *Au. pullulans* (80°C) [7] and *Ta. emersonii* enzyme (78°C) [19], and higher than that of the enzymes from *Tr. harzianum* (70°C) [20], *As. sojae* (60°C) [9], *H. grisea* (50°C) [1], *As. terreus* (40°C) [4], and *Bacillus* (37°C) [22]. The optimum temperature of the present enzyme was very different from that (50°C) of the extracellular *Au. pullulans* enzyme [17]. The enzyme was stable at 80°C for 15 min, retaining 95% of its original activity. The present enzyme was more stable than the extracellular enzyme from *Au. pullulans* [7], which retained half of its original activity at 75°C after 15 min.

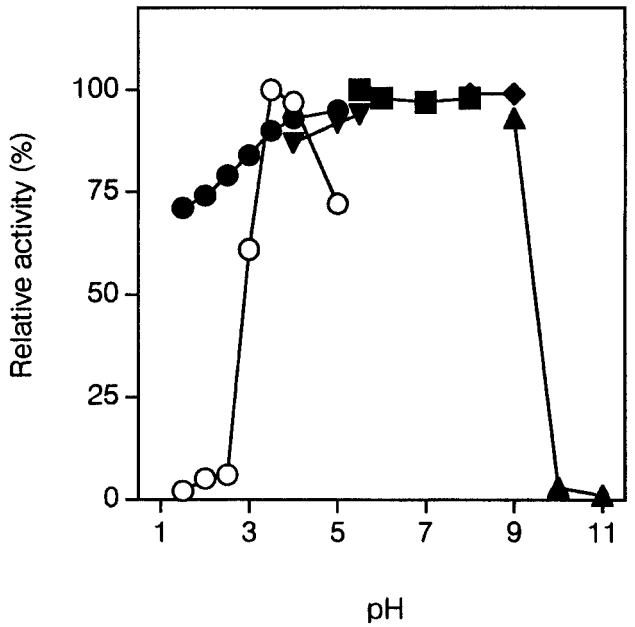

Figure 2 The effect of pH on the activity (open symbols) and stability (closed symbols) of β-xylosidase from *Aureobasidium*. The activities were measured after 3 h incubation at each pH and 25°C in acetete–HCl buffer (acidic pH, ●), acetate buffer (acid to neutral pH, ▼), phosphate buffer (neutral pH, ■), Tris–HCl buffer (neutral to alkaline pH, ◆) and glycine–NaOH buffer (alkaline pH, ▲) according to the method described in *Materials and Methods*.

Substrate specificity of the enzyme

Substrate specificity of the present enzyme was investigated (Table 2). The enzyme hydrolyzed PNPX and smaller xylo-oligosaccharides such as xylobiose and xylotriose. The present enzyme, however, did not hydrolyze the glycosidic linkages of polysaccharides. It is suggested that the enzyme has a specificity for PNPX similar to the *Bacillus* enzyme [22].

Table 2 Substrate specificity of β-xylosidase from *Aureobasodium*

Substrate	Relative activity (%)
p-Nitrophenyl-β-D-xyloside (5 mmol l^{-1})	100
p-Nitrophenyl-β-D-glucoside (5 mmol l^{-1})	2.5
p-Nitrophenyl-β-D-galactoside (5 mmol l^{-1})	0
p-Nitrophenyl-α-D-xyloside (5 mmol l^{-1})	0
Methyl-β-D-xyloside (5 mmol l^{-1})	1.7
Methyl-β-D-glucoside (5 mmol l^{-1})	0
Xylobiose (2 mmol l^{-1})	11.3
Xylotriose (2 mmol l^{-1})	8.0
Cellobiose (5 mmol l^{-1})	0
Lactose (5 mmol l^{-1})	0
Sucrose (5 mmol l^{-1})	0
Maltose (5 mmol l^{-1})	0
Xylan (0.5%)	0
CM cellulose (0.5%)	0
Starch (0.5%)	0

Kinetic parameter of the enzyme

The Michaelis constant (K_m) and maximum velocity (V_{max}) toward PNPX were determined from a Lineweaver–Burke plot. The K_m was found to be 2.0 mmol l^{-1} and the V_{max} was 0.94 mmol min^{-1} mg^{-1} protein. From these data, molar activity (k_{cat}) and catalytic efficiency ($k_{cat}K_m^{-1}$) were calculated to be 5.5×10^3 s^{-1} and $2.8\times10^6 s^{-1}$ mol^{-1}, respectively.

The wide range of K_m and V_{max} values of microbial β-xylosidase toward PNPX has been reported by many investigators. The values of the present enzyme were similar to those of the

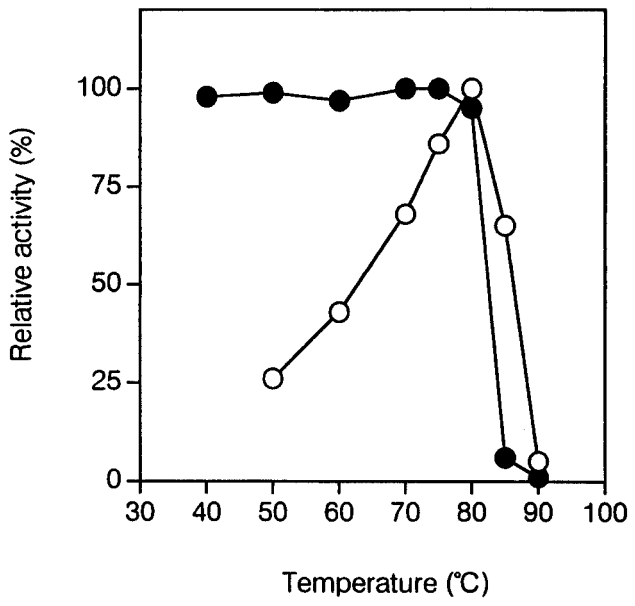

Figure 3 The effect of temperature on the activity (open symbols) and stability (closed symbols) of β-xylosidase from *Aureobasidium*. The activities were measured after at pH 6 after 15-min incubation at each temperature as described in *Materials and Methods*.

Table 3 Effect of various chemicals on the activity of β-xylosidase from *Aureobasodium*

Chemicals (1 mmol l^{-1})	Relative activity (%)
SH reagents	
p-Chloromercuribenzoic acid	106
Monoiodoacetic acid	53
Sodium arsenate	102
Sodium arsenite	101
Reducing reagents	
2-Mercaptoethanol	97
Hydrazine	52
Dithiothreitol	101
Chelating reagents	
Sodium azide	100
Ethylenediaminotetraacetic acid	40
Nitrilotriacetic acid	52
Sodium citrate	98
Detergents	
Sodium dodecyl sulfate	82
Triton X-100	102
Benzalkonium chloride	43
Control	100

Residual activities were measured after 24 h incubation in McIlvain buffer containing 1 mmol l^{-1} of each chemical.

Table 4 Effect of various metal ions on the activity of β-xylosidase from *Aureobasodium*

Metal ion (1 mmol l^{-1})	The number in the Periodic Table	Relative activity (%)
Na^{+}	(1)	103
K^{+}	(1)	106
Cs^{+}	(1)	99
Mg^{2+}	(2)	97
Ca^{2+}	(2)	105
Ba^{2+}	(2)	101
Mn^{2+}	(7)	93
Fe^{2+}	(8)	27
Co^{2+}	(9)	99
Ni^{2+}	(10)	99
Cu^{2+}	(11)	10
Ag^{+}	(11)	98
Zn^{2+}	(12)	93
Cd^{2+}	(12)	63
Hg^{2+}	(12)	1
Al^{3+}	(13)	38
Pb^{2+}	(14)	6
Control		100

Residual activities were measured after 24-h incubation in PIPES (piperazine-*N*,*N*-bis(2-etanesulfonic acid)/NaOH) buffer (40 mmol l^{-1}, pH 6.1) containing 1 mmol l^{-1} of metal ion.

enzyme from *P. verruculosum* (K_m 1.7 mmol l^{-1}; V_{max} 630 mmol min^{-1} mg^{-1} protein) [5]. The K_m value (0.43 mmol l^{-1}) of the extracellular *Au. pullulans* enzyme [7] was lower than that of the present enzyme. The catalytic efficiency of the present enzyme was higher than that of enzymes from *Agrobacterium tumefaciens* (5.8×10^3 s^{-1} mol^{-1}) [20] and *Ta. emersonii* (3.3×10^3 s^{-1} mol^{-1}) [19]. From the above results, it is suggested that the present enzyme has superior catalytic efficiency.

Effect of various chemicals and metal ions on enzymatic stability

The effects of typical organic inhibitors (Table 3) and metal ions (Table 4) on enzyme stability were investigated. The enzyme was inhibited by monoiodoacetic acid (53% of the original activity), hydrazine (52%), chelating reagents such as ethylenediaminetetraacetic acid (40%) and nitritoacetic acid (52%), and benzalkonium chloride (43%).

The enzyme was inhibited strongly by metal ions of the transition elements such as iron (group 8, 27% of the original activity), copper (group 11, 10%), mercury (group 12, 1%), aluminum (group 13, 38%), and lead (group 13, 6%), and less by cadmium (group 12, 63%). These elements generally combine with the functional groups of such amino acid residues as cysteine, tyrosine, lysine, and histidine. The pattern of the inhibition was similar to that of the *As. nidulans* enzyme [11].

The β-xylosidase activity of *Tr. harzianum* [21] and *H. grisea* [1] was enhanced by mercury and iron ions, respectively. However, no activation effect of these ions was observed on the present enzyme.

In the present paper, we investigated the characteristics of purified cell-associated β-xylosidase from *Aureobasidium* and compared them to those of the enzymes from other sources. We noted the superior stability, the catalytic efficiency, and the differences of the present enzyme from extracellular *Au. pullulans* enzymes.

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