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Purification and characterization of an extracellular β-xylosidase from a newly isolated *Fusarium verticillioides*

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An extracellular β-xylosidase from a newly isolated *Fusarium verticillioides* (NRRL 26518) was purified to homogeneity from the culture supernatant by concentration by ultrafiltration using a 10,000 cut-off membrane, ammonium sulfate precipitation, DEAE Bio-Gel A agarose column chromatography and SP-Sephadex C-50 column chromatography. The purified β-xylosidase (specific activity, 57 U/mg protein) had a molecular weight (mol. wt.) of 94,500 and an isoelectric point at pH 7.8. The optimum temperature and pH for action of the enzyme were 65°C and 4.5, respectively. It hydrolyzes xylobiose and higher xylooligosaccharides but is inactive against xylan. The purified β-xylosidase had a K_m value of 0.85 mM (*p*-nitrophenol-β-D-xyloside, pH 4.5, 50°C) and was competitively inhibited by xylose with a K_i value of 6 mM. It did not require any metal ion for activity and stability. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 241–245.

Keywords: β-xylosidase; *Fusarium verticillioides*; xylobiose; xylooligosaccharides; xylan

Introduction

β-Xylosidase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.37), which hydrolyzes xylobiose and short-chain xylooligosaccharides from the nonreducing end to xylose, is essential for complete breakdown of xylans. It plays an important role in relieving the end product inhibition of endoxylanases. The enzyme is part of most microbial xylanolytic systems. The β-xylosidase is cell associated in most bacteria and yeast, but it is found free in the culture media of some fungi [18].

Corn fiber xylan is a complex heteroxylan containing β-(1,4)-linked xylose residues. This backbone is highly substituted with monomeric side chains of arabinose or glucuronic acid linked to O-2 and/or O-3 of xylose residues, and also by oligomeric side chains containing arabinose, xylose and sometimes galactose residues [19]. It is highly branched with a high level of ferulic acid (5%) esterified to it as well as a high level of cross-linking through diferulic acid bridges between the heteroxylan chains [20]. Recently, we demonstrated that xylan in corn fiber is highly resistant to enzymatic degradation and that commercial hemicellulase preparations are not effective in degrading corn fiber xylan [17]. The author screened 132 soil samples surrounding decaying corn and wood for the purpose of isolating an organism that can produce enzymes for saccharification of corn fiber xylan. In a previous paper, the author has reported the production, purification and properties of xylanase from the newly isolated fungal strain *Fusarium verticillioides* (NRRL 26518) capable of utilizing corn fiber xylan as a growth substrate [16].

Only a few reports are available on the purification and characterization of β-xylosidase from microbial sources [18]. To the author's knowledge, no report is available on the purification and characterization of β-xylosidase from any *Fusarium* sp. This paper reports on the purification and properties of an extracellular β-xylosidase from the newly isolated *F. verticillioides* (NRRL 26518).

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Materials and methods

Materials

Oat spelt xylan, birch wood xylan, xylose, all aryl-glycosides and molecular weight (mol. wt.) markers for gel filtration were purchased from Sigma Chemical, St. Louis, MO. Wheat arabinoxylan, xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4) and xylopentaose (X_5) were from MegaZyme, North Rocks, Australia. Mol. wt. markers and precast gels for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), DEAE Bio-Gel A agarose, Bio-Gel A-0.5m gel, and the Aminex HPX-87C column for high-pressure liquid chromatography (HPLC) were obtained from Bio-Rad Laboratories, Hercules, CA. SP-Sephadex C-50, Mono P, PBE-94 and Polybuffer 96 were from Amersham Pharmacia Biotech, Piscataway, NJ.

Cultivation and enzyme production

The newly isolated fungal strain was deposited in ARS Culture Collection, Peoria, IL and was designated as *F. verticillioides* NRRL 26518 [16]. The medium used for seed culture and enzyme production was YMP (yeast extract, malt extract and peptone, 0.3% w/v each) with oat spelt xylan (1%, w/v) which was sterilized (121°C, 15 min) separately. The pH of the medium was adjusted to 5.0 with 1 M HCl before inoculation. A 125-ml Erlenmeyer flask containing 50 ml of medium was inoculated with a loopful of cells taken from a stock slant and incubated at 30°C on a rotary shaker (200 rpm) for 2 days. Shake flasks (1-l Erlenmeyer flasks containing 400 ml medium) were inoculated with 8 ml of this culture and cultivated on a rotary shaker (200 rpm) at 30°C. After 4 days, when β-xylosidase production reached maximum, the cells

were removed from the culture broth by centrifugation (18,000×*g*, 20 min). The resulting supernatant solution was used as the crude enzyme preparation.

Enzyme assay

β-Xylosidase activity was assayed in a reaction mixture (1 ml) containing 2 mM *p*-nitrophenyl β-D-xyloside (*p*NPβX), 50 mM acetate buffer, pH 4.5 and appropriately diluted enzyme solution. After incubation at 50°C for 30 min, the reaction was stopped by adding ice-cold 0.5 M Na_2CO_3 (1 ml) and the color that developed as a result of *p*-nitrophenol (*p*NP) liberation was measured at 405 nm. One unit (U) of β-xylosidase activity was defined as the amount of enzyme that liberates 1 μmol *p*NP per minute in the reaction mixture under these assay conditions.

Purification of β-xylosidase

All purification steps were performed at 4°C, unless otherwise stated.

Ammonium sulfate treatment: The culture broth (6750 ml) was concentrated by ultrafiltration with a stirred cell (Model 202, Amicon, Beverly, MA) equipped with a PM 10 membrane under nitrogen pressure of 20 psi. The concentrated broth (1825 ml) was then treated with ammonium sulfate (80% saturation) and left overnight. The precipitate was collected by centrifugation at 48,000×*g* for 30 min, dissolved in 50 mM Tris–HCl, pH 8.6 and then dialyzed overnight against the same buffer.

DEAE Bio-Gel A agarose column chromatography: The dialyzed enzyme solution was loaded on a DEAE Bio-Gel A agarose column (2.5×26 cm) pre-equilibrated with 50 mM Tris–HCl, pH 8.6. The column was washed extensively with the same buffer and eluted first with a gradient of 0–0.5 M NaCl in the same buffer (250 ml each) and then with 50 mM Tris–HCl, pH 8.6 plus 0.5 M NaCl (300 ml). β-Xylosidase was eluted as a single active enzyme peak. Active fractions were pooled, concentrated by ultrafiltration (PM 10 membrane) and dialyzed overnight against 50 mM acetate buffer, pH 4.5.

SP-Sephadex C-50 column chromatography: The dialyzed enzyme solution was applied to a SP-Sephadex column (2.5×10 cm) pre-equilibrated with 50 mM acetate buffer, pH 4.5. The column was washed extensively with the same buffer and eluted with a gradient of 0–0.5 M NaCl in the same buffer (150 ml each). The β-xylosidase activity eluted as a single protein and enzyme peak. The active enzyme fractions were pooled, concentrated by ultrafiltration using a PM 10 membrane and dialyzed overnight against 50 mM acetate buffer, pH 5.0. The dialyzed enzyme solution was used as purified β-xylosidase for subsequent studies.

Analytical methods

Protein was estimated by the method of Lowry *et al* [12] with bovine serum albumin as the standard. Protein in column effluents was monitored by measuring absorbance at 280 nm. SDS-PAGE was performed on a 7.5% gel according to Laemmli [11]. The apparent mol. wt. of the native enzyme was determined by gel filtration on Bio-Gel A-0.5m as described by Andrews [3], using apoferritin (mol. wt. 443,000), sweet potato β-amylase (200,000), yeast alcohol dehydrogenase (150,000), bovine serum albumin (66,000) and ovalbumin (45,000) as standard proteins. The isoelectric point (pI) of β-xylosidase was determined by chromatofocusing, performed in a PBE-Mono P anion exchange resin, packed in a 1.0×30.0-cm column. An enzyme sample in 25 mM ethanolamine with 10% glycerol, pH 9.4, was applied to the column, which was pre-equilibrated with the same buffer. Proteins were eluted with diluted (1:10) Polybuffer 96, pH 6.0. The K_m value was determined by the double-reciprocal plot method of Lineweaver–Burk using the KINET software program. The products of xylooligosaccharide (X_2–X_5) hydrolysis were analyzed by HPLC (Spectra-Physics, San Jose, CA) using an ion-moderated partition chromatography column (Aminex HPX-87C). The column was maintained at 85°C, and the sugars were eluted with Milli-Q (Millipore, Bedford, MA) deionized water at a flow rate of 0.6 ml/min. Peaks were detected by refractive index and identified and quantified by comparison to retention times of authentic standards (xylose, X_2–X_5). Reducing sugar release from xylan substrates was measured by the dinitrosalicylic acid method [14].

Results

Purification of β-xylosidase

An extracellular β-xylosidase was purified to homogeneity from the culture filtrates of *F. verticillioides* grown on oat spelt xylan. A summary of the purification procedures is presented in Table 1. Upon SDS-PAGE of the purified enzyme a single band was visualized when stained with Coomassie Brilliant Blue (Figure 1). The final purification resulted in a yield of 6.55% of the activity and 0.004% retention of total protein and a 1425-fold increase in specific activity (Table 1).

Characterization of β-xylosidase

Molecular weight: The apparent mol. wt. of the native β-xylosidase estimated by gel filtration on Bio-Gel A-0.5m was

Table 1 Purification of β-xylosidase from *F. verticillioides* NRRL 26518

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification (fold)
Culture supernatant	7763	290	0.04	100	1
Concentration by ultrafiltration (PM 10)	6670	303	0.05	104	1
Ammonium sulfate (80% sat.)	1860	243	0.13	84	3
DEAE Bio-Gel A agarose	78	88	1.13	30	28
SP-Sephadex C-50	0.3	19	57	7	1425

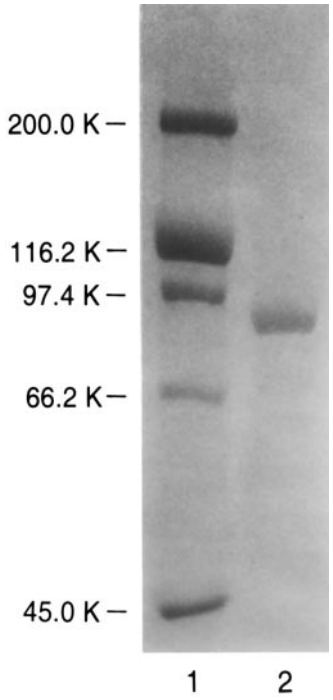

Figure 1 SDS-PAGE of purified β-xylosidase from *F. verticillioides* NRRL 26518. The enzyme (~20 μg protein) was electrophoresed at pH 8.3 on a 7.5% acrylamide gel and stained with Coomassie Brilliant Blue R-250. 1, mol. wt. standards; 2, purified β-xylosidase. The standards were myosin (200,000); β-galactosidase (116,250); phosphorylase B (97,400); bovine serum albumin (66,200); and ovalbumin (45,000).

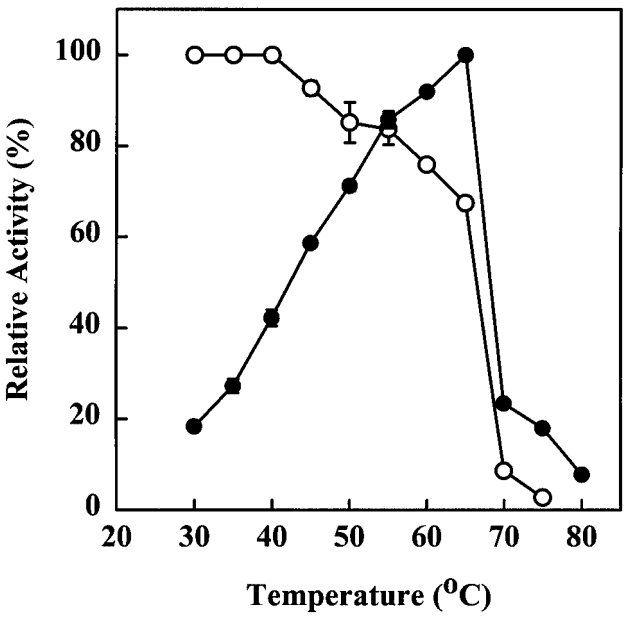

Figure 2 Effect of temperature on stability (○) and activity (●) of purified β-xylosidase from *F. verticillioides* NRRL 26518. For stability, the enzyme solution in acetate buffer (50 mM, pH 5.0) was incubated for 30 min at various temperatures, and then the residual enzyme activities were assayed by the standard method. For activity, enzyme activity was assayed at various temperatures by the standard assay method. Enzyme used, 18 mU/ml.

79,500. By SDS-PAGE analysis, the mol. wt. of the enzyme was about 94,500 (Figure 1) suggesting that the β-xylosidase was a monomeric protein. The pI of the enzyme as determined by chromatofocusing was 7.8.

pH and temperature dependence: The thermostability and thermoactivity of the purified β-xylosidase are shown in Figure 2. The purified enzyme (18 mU/ml; 0.3 μg protein/ml) in 50 mM acetate buffer, pH 5.0 was fairly stable up to 40°C for 30 min. It exhibited optimal activity at 65°C under the assay conditions used. As shown in Figure 3, the enzyme was quite stable at pH 4.5–6.0 with only 8% activity remaining at pH 3.0 and 41% activity remaining at pH 8.0 (1 h at 40°C). It displayed optimum activity at pH 4.5 with no activity at pH 3.0 and 11% activity at pH 8.0.

Substrate specificity and kinetic analysis: The degradation of various xylooligosaccharides (X_2–X_5) by purified β-xylosidase was followed by analyzing the reaction products by HPLC (Table 2). The enzyme released xylose from all substrates. The rate of xylose release increased with the increase of chain length of the xylooligosaccharides (X_2–X_5). The purified β-xylosidase did not possess any activity against *p*NP-α-L-arabinofuranoside, *p*NP-β-D-galactoside, *p*NP-α-D-glucoside and *p*NP-β-D-glucoside. It was not able to degrade oat spelt xylan, birch wood xylan and wheat arabinoxylan. The rate dependence of the enzymatic reaction on the *p*NPβX concentration at pH 4.5 and 50°C followed Michaelis–Menten kinetics. A reciprocal plot showed an apparent K_m of 0.85 mM for hydrolysis of *p*NPβX by the enzyme. Enzyme activity was competitively inhibited by xylose with a K_i of 6 mM. No transfer product from

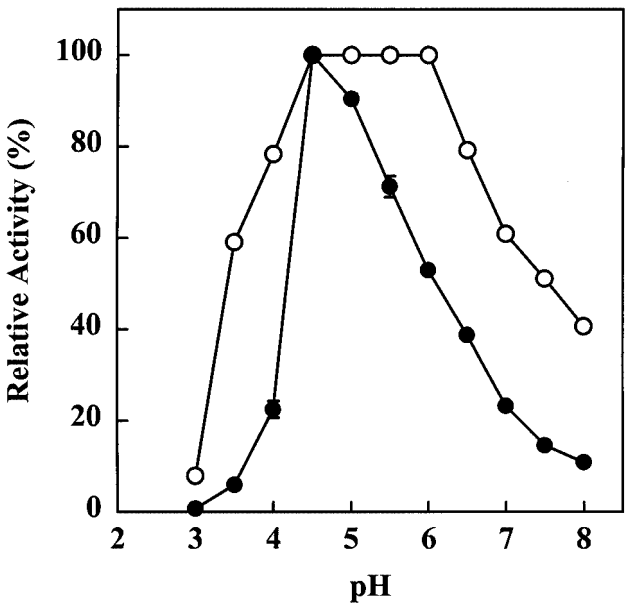

Figure 3 Effect of pH on stability (○) and activity (●) of purified β-xylosidase from *F. verticillioides* NRRL 26518. For stability, the enzyme solutions in 50 mM citrate–phosphate buffer at various pH values were incubated for 1 h at 40°C. After adjustment of pH, residual activity was assayed by the standard method. Enzyme activity was assayed by the standard assay method by changing the buffer to obtain the desired pH. Buffer used, 50 mM citrate–phosphate, pH 3.0–8.0. Enzyme used, 18 mU/ml.

Table 2 Xylooligosaccharide hydrolysis by purified β-xylosidase from *F. verticillioides* NRRL 26518

Substrate (1%, w/v)	Xylose release[a] (%)	Product
Xylobiose	100	X_1
Xylotriose	120	X_2, X_1
Xylotetraose	123	X_3, X_2, X_1
Xylopentaose	130	X_3, X_2, X_1

At pH 4.5, 50 °C, 2 h reaction. Enzyme used: 0.3 U/ml. X_1, xylose; X_2, xylobiose; X_3, xylotriose; X_4, xylotetraose; X_5, xylopentaose.
[a]Based on the amount of xylose release from X_2 which was considered as 100%.

xylose (20%) was detected when the purified enzyme (2.66 U/ml) was incubated at 50°C, pH 5.0 for 96 h.

Effect of metal ions and reagents: The influence of certain potential inhibitors or activators on β-xylosidase activity was studied. The enzyme did not require Ca^{2+}, Mg^{2+}, Mn^{2+} (each at 5 mM) or Co^{2+} (0.5 mM) for activity. Enzyme activity was not affected by ethylenediaminetetraacetate (EDTA, 10 mM), dithiothreitol (DTT, 10 mM) or by *p*-chloromercuribenzoic acid (*p*CMB, 0.2 mM, pH 6.5).

Discussion

Although reports are available on the purification and properties of xylanase from *Fusarium oxysporium* [1,4], this is the first report on the purification and characterization of β-xylosidase from a *Fusarium* sp. The purified β-xylosidase from *F. verticillioides* had a specific activity of 57 U/mg protein (Table 1). In comparison, some fungal-purified β-xylosidases had very low specific activities (U/mg protein): 2.4 (*Trichoderma lignorum*), 3.29 (*Aspergillus carbonarius*), 3.42 (*Trichoderma harzianum*) and 0.9 (*Neocallimastrix frontalis*) [6,8,9,23]. The purified β-xylosidase from *F. verticillioides* exits in a single form as no other form of the enzyme was detected during purification. The β-xylosidase from *A. pulverulentus*, *Neocallimastrix patriciarum* and *Penicillium wortmanni* showed two, two and four forms, respectively [13,21,24]. The β-xylosidase from *F. verticillioides* appears to be a monomeric protein (mol. wt. 94,500) with a pI of 7.8. The β-xylosidase from *Talaromyces emersonii* had a pI at pH 8.9 [22]. In contrast, most β-xylosidases reported to date have acidic pI [18]. The β-xylosidase from *F. verticillioides* appears to act exclusively by removing xylose from xylooligosaccharides. The rate of release of xylose from xylooligosaccharides (X_2–X_5) by the purified enzyme increased with increasing chain length (Table 2). In this respect, this β-xylosidase is similar to the β-xylosidase from *T. lignorum* [8]. The purified β-xylosidase was free from α-L-arabinofuranosidase (EC 3.2.1.55), β-galactosidase (EC 3.2.1.23), α-glucosidase (EC 3.2.1. 20) and β-glucosidase (EC 3.2.1.21) activities. It was also free from xylanase (EC 3.2.1.8) activity as the purified enzyme did not degrade polymeric xylans such as oat spelt xylan, birch wood xylan and wheat arabinoxylan. In contrast, the purified β-xylosidase from *Trichoderma reesei* is a mutifunctional β-D-xylan xylohydrolase having α-L-arabinofuranosidase activity and ability to form xylose from xylan [7]. β-Xylosidase is an important enzyme in the conversion of xylobiose and other higher xylooligosaccharides to xylose. However, most β-xylosidases reported to date are inhibited by xylose [18]. The purified β-xylosidase of *F. verticillioides* NRRL 25618 is also competitively inhibited by xylose with a K_i value of 6 mM. In this respect, this enzyme is similar to β-xylosidases from *T. reesei* (K_i 2.4 mM) and *N. frontalis* (K_i 3.98 mM) [6,7]. The enzyme from *Humicola grisea* var. *thermoidea* was not inhibited by xylose which was tested only at 10 mM concentration [2]. The purified β-xylosidase of *F. verticillioides* NRRL 25618 did not require any metal ion for activity and stability. Comparative properties of some fungal β-xylosidases are presented in Table 3. For practical purposes, it is essential that a xylose-tolerant β-xylosidase is developed in order to make enzymatic saccharification of alkali pretreated xylan substrates to fermentable sugars for production of fuel ethanol and value-added fermentation products a commercial success.

Table 3 Comparative properties of some fungal β-xylosidases

Fungus		Molecular weight	pI	Optimum temperature (°C)	Optimum pH	Inhibition by xylose (K_i, mM)
F. vercillioides [this work]		94,500	7.8	65	4.5	6.0
A. carnonarius [9]		100,000	4.4	60	4.0	1.9
A. nidulans [10]		180,000	3.4	50	5.0	Inhibited by xylose
A. pulverulentus [21]	β-Xyl I	180,000	4.7	60	2.5–3.5	–
	β-XylII	190,000	3.5	60	4.0–5.0	–
A. phoenicis [15]		132,000	3.7	75	4.0–4.5	–
Au. pullulans [5]		224,000	–	80	4.5	Inhibited by xylose
H. grisea var. *thermoidea* [2]		43,000	4.0	50	6.0	–
N. frontalis [6]		150,000	4.6	37	6.4	3.98
N. patriciarum [24]	I	39,500	4.7	50	6.0	–
	II	150,000	4.7	40	6.0	–
P. wortmanni [13]	1	110,000	3.7	55–65	3.0–4.5	–
	2	195,000	4.28	55–65	3.0–4.5	–
	3	210,000	4.6	55–65	3.0–4.5	–
	4	180,000	4.8	55–65	3.0–4.5	–
Ta. emersonii [22]		181,000	8.9	60	2.5	–
T. harzianum [23]		60,000	–	70	4.0–4.5	Inhibited by xylose
T. reesei [7]		100,000	4.7	60	4.0	2.4

–, Not reported.

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