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The alkaline xylanase III from *Fusarium oxysporum* F3 belongs to family F/10

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Abstract

Xylanase III from *Fusarium oxysporum* F3 was purified to homogeneity by ion-exchange chromatography and gel filtration. The enzyme has a molecular mass of 38 kDa, an isoelectric point of 9.5, and is maximally active on oat spelt xylan at pH 7 and 45 °C with a K_m of 0.8 mg/mL. The xylanase displays remarkable stability at pH 9.0. It is not active on xylotriose but hydrolyzes the 4-methylumbelliferyl glycosides of β-xylobiose and β-D-glucopyranosyl-(1 → 4)-β-D-xylopyranose, and to a lower extent 4-methylumbelliferyl β-cellobioside. When acted on xylooligosaccharides and xylan, analysis of reaction mixtures by high-pressure liquid chromatography shows preferred internal glycoside cleavage. Thus the purified enzyme appears to be a true *endo*-β-1,4-xylanase. Partial amino acid analysis of xylanase III shows high sequence homology with xylanases of family F/10. © 1997 Elsevier Science Ltd.

Keywords: Xylanase; *Fusarium oxysporum*; Enzyme purification; Partial amino acid sequence

1. Introduction

Hemicelluloses are found in almost all parts of green plant cell walls [1]. The enzymes acting on the xylan backbone of these heterogeneous polysaccharides are classified in two groups: *endo*-β-1,4-xylanases (EC 3.2.1.8, xylan xylanohydrolase) and *exo*-β-1,4-xylanases (EC 3.2.1.37, D-xylan xylanohydrolase). Endoxylanases prefer the internal β-(1 → 4) bonds of xylan, and have wide commercial significance being used in industrial processes such as bioconversion of lignocellulose-derived sugars into fuels, biopulping, bread making, and clarification of beer and juices [2]. *Fusarium oxysporum* F3 produces several xylanases, and this work focuses on xylanase III. Xylanases and cellulases have been classified into several families on the basis of sequence similarities and xylanases belong to either family F or family G [3]. These families correspond

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to families 10 and 11 of the glycosyl hydrolase classification [4].

2. Experimental

Materials and methods.—(a) Chemicals. Sephacryl S-200 and SP-Sepharose were purchased from Pharmacia, Sweden. Xylooligosaccharides (dp = 2–6) were from Megazyme, Australia. Soluble birchwood xylan was from Roth, Germany. 4-Methylumbelliferyl glycosides were synthesized as described [5]. All the remaining substrates were AnalaR grade from Sigma, USA.

(b) Microorganism and culture conditions. F. oxysporum strain F3, used in the present study, was isolated from cumin [6]. The fungus was cultured in 500-mL Erlenmeyer flasks for 4 days at 30 °C and pH 8.0 using a minimal medium of 2% (w/v) corn cobs as carbon source and 1% (w/v) ammonium phosphate as nitrogen source (200 mL) [7].

Crude enzyme production.—Mycelium was filtered (Whatman No. 4 paper), and the centrifuged fluid culture (5 L) was concentrated 100-fold by ultrafiltration (Amicon PM 10), dialyzed against distilled water, and stored at −20 °C.

Enzyme purification.—(a) Ion-exchange chromatography. One hundred mg of crude enzyme in 50 mM bicine buffer, pH 9, were loaded onto a SP-Sepharose column (1 × 15 cm), equilibrated with the same buffer. The column was first washed with 40 mL buffer and then a linear gradient of 0–0.5 M NaCl in 100 mL of the same buffer was applied, at a flow rate of 60 mL/h. Fractions of 4 mL each were collected and assayed for xylanase activity; combined fractions were concentrated by dialysis (SM 13200 collodion bags, Sartorius).

(b) Gel filtration. A column (2.5 × 100 cm) of Sephacryl S-200, pre-equilibrated with 50 mM bicine buffer, pH 9, was used, and elution was performed with the same buffer (flow rate, 60 mL/min).

Biochemical characterization of purified xylanase. —(a) Enzyme assay. Endo-β-1,4-xylanase activity was assayed in 50 mM sodium phosphate buffer, pH 7, at 50 °C for 15 min using 1.0% oat spelt xylan as a substrate. The release of reducing sugars was determined by the 3,5-dinitrosalicylic acid (DNS) method [8] with one unit defined as the amount of enzyme liberating 1 μmol of reducing sugars per minute.

The action of purified enzymes on CMC (1%), Avicel (1%), *p*-nitrophenyl β-D-xylopyranoside (PNPX; 2 mM) and *p*-nitrophenyl β-D-glucopyranoside (PNPG; 2 mM) at pH 7.0 and at 50 °C was studied by measuring either the reducing sugars or *p*-nitrophenol released from respective substrates as described [9,10].

Reactions with the 4-methylumbelliferyl glycosides of β-lactose (MUL), β-D-glucose (MUG), β-D-xylose (MUX), β-cellobiose (MUG_2), β-xylobiose (MUX_2), and β-D-glucopyranosyl-(1 → 4)-β-D-xylopyranose (MUGX) (W. Nerinckx et al., unpublished results) were set up in microtiter dishes with 100 μM substrate, 0.05 M sodium phosphate buffer, pH 7.0, and 1 μg of enzyme. After 5 and 30 min at 35 °C the plates were UV transilluminated and photographed.

(b) Enzymatic hydrolysis of xylan and xylooligosaccharides. Hydrolysis products of oat spelt xylan were analyzed by high-performance anion-exchange chromatography using a Dionex DX500 pulsed amperometric detection system (HPAE-PAD) (Dionex, Sunnyvale, CA, USA), with D-xylose, xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose as references. The reaction mixtures (1 mL of 100 mM sodium phosphate buffer, pH 7) containing 1 μg of xylanase III and 1 mg xylan were incubated for 1 h at 37 °C. Similar mixtures and hydrolysis conditions were used to determine the hydrolysis products of 10 mM xylooligosaccharides (dp = 2–6).

(c) Determination of pH and temperature optima, pH-stability and thermostability. The optimum temperature was determined by assaying the enzyme activity at various temperatures (40–70 °C) for 15 min in 0.1 M potassium phosphate buffer, pH 7.0. The optimum pH was determined by measuring the activity at 40 °C over the pH region 4.0–10.0 using the following buffers: 0.1 M citrate-HCl (pH 4.0–6.0), 0.1 M potassium phosphate (pH 7.0–8.0), 0.1 M Tris-HCl (pH 9.0), and 0.1 M glycine-HCl buffer (pH 10.0). The enzyme pH-stability was determined after incubating the enzyme in the buffers described above for 24 h at 30 °C and by measuring the residual activity as above. The thermostability was determined by measuring the residual activity, after incubation of the purified endoglucanase (50 μg/mL) for 60 min at varying temperatures (30–65 °C), in buffer pH 7.0 in the absence of substrate.

(d) Determination of molecular mass. This was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [11].

(e) Determination of isoelectric point (pI). The p*I* was determined by isoelectric focusing-polyacrylamide gel electrophoresis (IEF-PAGE). This was per-

formed using the Multiphor IEF system (LKB) with Ampholine PAG plate pH 3.5–9.5 (Pharmacia).

(f) Amino acid analysis. Amino acid analysis was performed using a Perkin–Elmer amino acid analyzer (Perkin–Elmer Applied Biosystems, Foster City, CA). The protein was hydrolyzed in vacuo with 6 M HCl, in sealed tubes at 110 °C for 24 h. Elution profiles were recorded and peaks integrated.

(g) Enzyme digestion and peptide sequencing. Xylanase III (125 μg) was lyophilized and taken up in 250 μL 25 mM Tris-HCl, pH 7.8, and the digestion was performed with 10 μg endoproteinase Lys-C (*Lysobacter enzymogenes*, Boehringer Mannheim) for 17 h at 37 °C. The resulting peptides were separated by reversed-phase HPLC (RP-HPLC) on a C2/C18 column (3.2 × 30 mm, 0.3 μm) using the SMART system (Pharmacia). The flow rate was 100 μL/min and the gradient applied was 0.05% CF_3CO_2H in H_2O to 0.04% CF_3CO_2H in 30:70 H_2O-acetonitrile over a period of 80 min. Peptides were detected by their absorbances at 220 and 280 nm and, after collection, fractions were concentrated in a vacuum centrifuge. Peptide sequencing was performed on a 476A pulsed liquid sequenator, equipped with an on-line PTH-amino acid analyzer (Perkin–Elmer Applied Biosystems, Foster City, CA).

(h) Protein estimation. The protein concentration was determined by the bicinchoninic acid method as described in Pierce instruction manual No. 23235X using bovine serum albumin as a standard. Protein concentration in column effluents was monitored spectrophotometrically at 280 nm.

3. Results and discussion

Enzyme purification.—When grown on corn cobs, *F. oxysporum* produces several xylanases [12]. Xylanase III was presently purified to apparent homogeneity. Upon ion-exchange chromatography, two activity peaks (fractions 4–7 and 18–23) appeared and the concentrated second fraction (total volume 24 mL) was further purified by gel filtration. Two xylanase active fractions (fractions 63–69 and 82–90) were obtained, of which the first fraction (total volume 28 mL) corresponds to xylanase III. The summary of the purification is given in Table 1. A single band was observed upon SDS-PAGE (not shown) and its molecular mass was found to be 38 kDa. This value is in accordance with those reported for purified fungal xylanases belonging to family F/10, e.g.

Table 1
Summary of the purification of xylanase III from *F. oxysporum*

Purification step	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U mg protein^{-1})
Culture filtrate	135	33110	100	331
SP-Sepharose	24	9108	9	1012
Sephacryl S-200	28	7315	6	1219

from *Penicillium chrysogenum* [13], *Aspergillus awamori* [14], and *Filobasidium floriformei* [15]. Electrofocusing (pH 3.0–9.5) of the purified xylanase yielded a p*I* of about 9.5 (not shown). This is in contrast with the acidic p*I*'s of several other fungal xylanases but corresponds to those of *Bipolaris sorokiniana* [16], *Ceratocystis paradoxa* [17], *Trichoderma pseudokoningii* [18], and *Trichoderma viride* [19].

Enzyme characterization.—The xylanase pH optimum is at 6–8, and 72% of the maximum activity remains at pH 9. Being stable in a wide pH range (7–9), 78% of the activity is retained at pH 10 after 24 h at 20 °C. Thus the enzyme has a higher pH optimum and is more stable in alkaline conditions than most other fungal xylan-degrading enzymes. However, alkali-tolerant xylanases from *Aspergillus fisheri* [20], *Cephalosporium sp.* [21] and *Nocardiopsis dassonvillei* [22] have also been characterized. As for these enzymes, the high activity and stability in alkaline conditions of *F. oxysporum* xylanase may be advantageous in biotechnological applications especially in the treatment of alkaline pulp.

Measured against oat spelt xylan as a substrate, the enzyme's properties, such as temperature optimum (40–50 °C) and thermostability (73% retained activity at 50 °C after 1 h at pH 7), are similar to those reported for xylanases from other mesophilic fungi [23]. The Michaelis–Menten constant was determined at xylan concentrations ranging from 0.1 to 2.0 mg/mL. From a Lineweaver-Burk plot, the K_m is found to be 0.8 mg/mL and the V_{max} is 1.22 mmol (mg protein)$^{-1}$ min^{-1}, competing favorably with those of other xylanases.

Substrate specificity.—The activity of the purified enzyme towards several substrates is presented in Table 2. Xylanase III was most active on oat spelt xylan, the activity on carboxymethylcellulose and microcrystalline cellulose (Avicel) being extremely low.

Table 2
Relative activities of xylanase III from *F. oxysporum* towards different substrates (1% w/v)

Substrate	Xylanase III activity (%)
Oat spelt xylan	100
Birch wood xylan	37
Larch wood xylan	31
4-*O*-Methylglucuronoxylan	69
Carboxymethylcellulose	nil
Microcrystalline cellulose	nil

Activities on the methylumbelliferyl derivatives are shown qualitatively in Fig. 1. The presence of an aryl group (MU) renders some glycosides hydrolysable; thus, MUX_2, MUGX and to a lower extent MUG_2, are substrates. The specificity of the enzyme is in this respect very similar to that of a *Cellvibrio gilvus* xylanase [24]. Like the xylanases belonging to family F/10 [25] the enzyme is active (be it on a much smaller degree) on aryl β-cellobiosides such as MUG_2 (Fig. 1).

Mode of action.—Examination of the hydrolysis products of oat spelt xylan and xylooligosaccharides indicate that the enzyme has an endo character (Table 3). Clearly the xylanase attacks mainly the internal glycosidic bonds releasing xylobiose and xylotriose. Like fungal xylanases from *Neocallimastix frontalis* [26], *Trichoderma reesei* [27], and *Aspergillus oryzae* [28], the presently studied enzyme is not active on xylotriose.

Amino acid composition and partial amino acid sequence.—The amino acid composition is reported in Table 4. Cellulases and xylanases, based on their amino acid sequence and on hydrophobic cluster

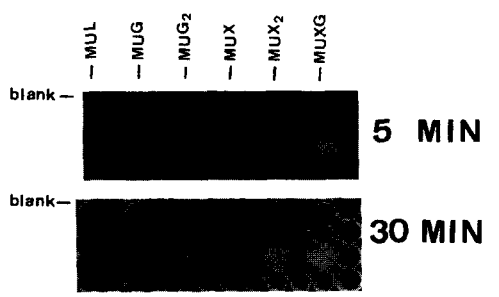

Fig. 1. Activity of purified xylanase III from *F. oxysporum* (1 μg) on 4-methylumbelliferyl glycosides (100 nM) of: β-lactose (MUL), β-D-glucose (MUG), β-D-xylose (MUX), β-cellobiose (MUG_2), β-xylobiose (MUX_2), and β-D-glucopyranosyl-(1 → 4)-β-D-xylopyranose (MUGX). Incubated at 35 °C and pH 7. Lane 1: blank; lane 3: test samples; lane 2 and 4: void.

Table 3
Hydrolysis products released from xylooligosaccharides and oat spelt xylan by xylanase III from *F. oxysporum*

Substrate	Molar ratio of product (%)					
	X_1	X_2	X_3	X_4	X_5	X_6
X_4	–	100				
X_5	6	47	42	5		
X_6	6	60	17	18	3	
Oat spelt xylan	6	56	25	7	4	2

For details, see Experimental section. Reaction time and temperature were 1 h and 37 °C, respectively. X_1, D-xylose; X_2, xylobiose; X_3, xylotriose; X_4, xylotetraose; X_5, xylopentaose; X_6, xylohexaose; xylobiose and xylotriose were not hydrolysed.

analysis, have been classified in a number of families [3,4]. The amino acid sequences of two Lys-C fragments obtained by partial proteolysis of xylanase III are presented in Fig. 2. A search for homologies with other proteins was made through the National Center for Biotechnology Information (NCBI) using the BLAST network service. Our partial sequencing data allow the assignment of xylanase III to family F/10 based on its analogy to several xylanases of this family (Fig. 2).

In a separate report the unequivocal presence of a cellulose-binding domain (CBD) has been demonstrated [29]. This represents the first example of a CBD-containing xylanase from fungal origin.

Table 4
Amino acid composition of xylanase III from *F. oxysporum*

Amino acid	Composition (mol %) [a]
Asp	10.93
Glu	7.68
Ser	2.16
Glu	8.52
His	1.88
Arg	5.43
Thr	7.27
Ala	13.36
Pro	6.55
Tyr	4.20
Val	6.83
Met	2.24
Ile	6.64
Leu	9.54
Phe	2.68
Lys	4.09
Cys	n.d.
Trp	n.d.

[a] n.d., not determined.

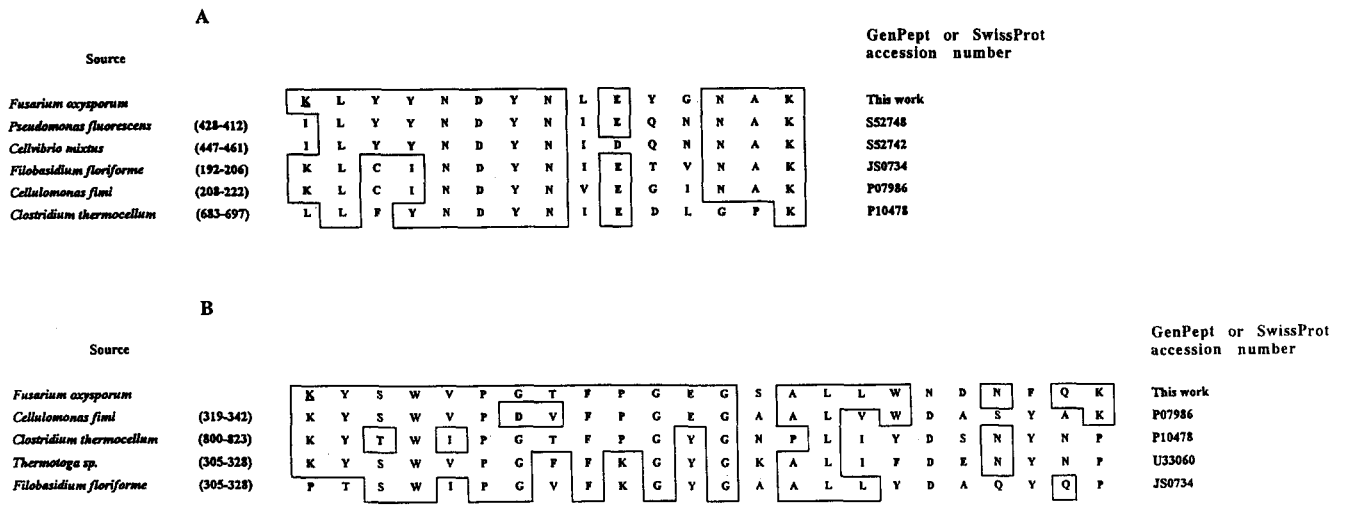

Fig. 2. Partial amino acid sequences of xylanase III from *F. oxysporum* and their comparison with equivalent sequences of xylanases in Family F/10 of glycanases. Residues with sequence identity or similarity in all sequences are boxed. (A) N-Terminal sequence of Lys-C fragment I. (B) N-Terminal sequence of Lys-C fragment II. *K* indicates where lysine is presumed to be present because of the cleavage by Lys-C endoproteinase.

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