

BASIC XYLANASES FROM THE FUNGAL TOMATO PATHOGEN *FUSARIUM OXYSPORUM* F.SP. *LYCOPERSICI*

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ABSTRACT

We purified four xylan-degrading enzymes (*endo*-xy-lanases: EC 3.2.1.8) produced *in vitro* by the fungal pathogen *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*). The xylanases were resolved by a combination of ion exchange and hydrophobic interaction chromatography, and preparative native-PAGE electrophoresis. They had molecular masses of 38, 34, 22 and 19 kDa, pI higher than 9.3, *endo*-cleavage mechanism and maximal activity at pH 6.0 and 45°C. Comparison of the reaction products showed differences in the cleavage mechanism of two of the xylanases.

In addition their role as possible elicitors of stress and defence responses was examined in preliminary assays. Xylanases from a variety of pathogenic and non-pathogenic fungi have been described as inducers of plant stress and defence responses. A role as elicitors of active defence mechanisms was suggested for the ethylene biosynthesis-inducing xylanase from *T. viride* (EIX) and EIX-like enzymes from a variety of pathogenic fungi. In spite of the homologies with the EIX from *T. viride* none of the *Fol* xylanases examined by us were antigenically related to it. Furthermore, they showed no EIX-like eliciting activity on cell culture suspensions or intact tomato leaflets in our tests. Therefore their role as recognition factors is not established in our experimental model.

Key words: CWDEs, elicitors, *Fusarium*.

INTRODUCTION

Fungal plant pathogens produce a range of enzymes capable of degrading components of plant cell walls (Cooper *et al.*, 1988). The aggressive role of these enzymes is most evident in interactions where macerating processes are important; nonetheless, they may also

play a prominent role in typically non-lytic diseases. Among them, pectin-degrading enzymes are the best studied by far, as related to fungal pathogenesis (for a review, see Alghisi and Favaron, 1995).

Several lines of evidence suggest however that hemi-cellulases (notably *endo*-xy-lanases, EC 3.2.1.8) may be significant in plant-pathogen interactions. They are not able to macerate tissues by themselves, especially in dicotyledons, where xylan polymers are much less abundant than in the cell wall of monocotyledons. These enzymes may not be massively produced in interactions between the dicots and their pathogens (Holden and Walton, 1992). Nevertheless, xylanases from several pathogenic and non-pathogenic fungi (notably the ethylene biosynthesis-inducing xylanase, EIX, from *Trichoderma viride*) have been studied as inducers of plant responses. Among these there are cell death, membrane damage, electrolyte leakage, MAP kinase activation, fatty acid glycosylation, and accumulation of ethylene, proteins related to pathogenesis, and phytoalexins (Farmer and Helgeson, 1987; Ishii, 1988; Bailey *et al.*, 1990; Bucheli *et al.*, 1990; Lotan and Fluhr, 1990; Bailey *et al.*, 1992; Avni *et al.*, 1994b; Moreau *et al.*, 1994; Chapman *et al.*, 1995; Anderson *et al.*, 1997; Nühse *et al.*, 2000). The xylanase activity of EIX is not required for elicitation (Sharon *et al.*, 1993; Enkerli *et al.*, 1999), and a high-affinity binding site for EIX has been detected on membranes from sensitive tobacco and tomato cells (Hanania and Avni, 1997). Furthermore, proteins (presumably xylanases) antigenically related to the well-known EIX from *T. viride* have been detected in culture filtrates of several phytopathogenic fungi including *Fusarium oxysporum* f.sp. *pisi* (Dean *et al.*, 1989).

Recently, an acidic *endo*- β -1,4-xylanase from *F. oxysporum* f.sp. *lycopersici* (*Fol*) has been purified and characterised [XYL1, molecular mass 40 kDa (Ruiz *et al.*, 1997)] and the genes (*xyl2* and *xyl3*) corresponding to two additional xylanases of about 36 and 42 kDa (Ruiz *et al.*, 1998) have been isolated. No effort has been made so far, though, to isolate and characterise basic xylanases from this fungus. These enzymes, on which our work focused, may be the functional homo-

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logues of eliciting xylanases from other non-pathogenic fungi. The present report describes the purification of some of the xylan-degrading enzymes produced *in vitro* by *Fol*, and preliminary results on their possible role in stress and defence responses in tomato plants.

MATERIALS AND METHODS

Fungal cultures. A *Fol* strain of tested pathogenicity was maintained on potato-dextrose agar, and inoculated to liquid mineral medium (Baker *et al.*, 1992) without glucose. Except when otherwise stated, for all preliminary experiments (as for the preparative cultures) 0.1% soluble xylan [obtained as in Doux-Gayat and Auriol (1976) from commercial birchwood xylan, Sigma] in mineral medium was used. Mycelium was harvested 3-4 days after inoculation; flasks were incubated in the dark at 25°C with shaking (100 rpm).

Electrophoresis, protein measurements and immunoblotting. SDS-PAGE was performed in 0.75, 1 or 1.5 mm thick minigels of 17.5 or 20% acrylamide for the resolving gel in a vertical electrophoresis apparatus (Hoefer SE250) according to Laemli (1970). Proteins were visualised by silver nitrate (Wray *et al.*, 1981). IEF-PAGE was performed on Phast System (Pharmacia) as described by Ferraris *et al.* (1996); protein concentration was determined by the Bradford method using γ -globulin as a standard. Proteins separated by SDS-PAGE were blotted by diffusion from minigels onto nitrocellulose membranes as recommended by Phast System (Pharmacia). Immunodetection was performed according to Johnson *et al.* (1984) using anti-EIX antibodies (1:5000 in blocking buffer) obtained as reported by Dean *et al.* (1989).

Spectrophotometric enzyme assays and zymograms. Xylanase activity was assayed by measuring the release of reducing sugars from xylan (Nelson, 1944). One enzymatic unit (U) is the amount of enzyme releasing 1 μ mol of reducing units per min from 0.01% soluble xylan in 50 mM Na-Acetate, pH 6.0 during a 10 min test at 45°C. When present, bars indicate the S.E. of the values obtained for two flasks per treatment and three measurements per flask. Xylanase activity after IEF- and native-PAGE was detected by the overlay technique described by Biely *et al.* (1985b). Briefly, RBB-xylan obtained according to the method of Biely *et al.* (1985a) was used as substrate in an agarose overlay (0.5% w/v in 200 mM Na-acetate buffer, pH 6.0). Active proteins are visualised as destained bands on the deep-blue background after washing off the fragments

of dye-coupled xylan detached from the embedded polymer by active xylanases.

Analysis of xylanase reaction products. Water-dialysed and lyophilised proteins (containing equal amounts of xylanase activity) from the different fractions obtained during purification were resuspended in 5 ml of xylan solution (3 mg ml⁻¹ in 50 mM acetate buffer, pH 6.0, and 0.01% NaN₃). Substrate alone was used as a control. One ml aliquots were collected at different times (5, 10, 20, 30 min; and 1, 3, 24 and 96 h) from the tubes incubated with shaking at 40°C. Xylanase reaction products were resolved by size-exclusion chromatography (SEC) on an HPLC system (Vari-an 5000 liquid chromatography) equipped with a 2 ml semipreparative loop (Rheodyne) and visualised by refractometry (TSP ReFractoMonitor IV).

Enzyme purification

Sample preparation and tangential flow ultrafiltration (UF). The fungus was grown for 3 days in shaken culture (100 rpm) on mineral medium containing 0.1% soluble xylan. Culture filtrates were cleared by centrifugation (17,700 g x 15 min) and the supernatant microfiltered through Millipore membranes (0.80 and 0.22 μ m). The cell-free filtrate (CF fraction) was subjected to two rounds of ultrafiltration in a tangential-flow apparatus (Millipore). Initial passage through a 30 kDa molecular mass cut-off membrane retained large molecules (retentate; UF30 fraction). Low mol. wt components passed into the filtrate, which was subsequently concentrated about 10-fold by ultrafiltration against a 3 kDa mol. mass cut-off cassette (UF3 retentate fraction). During this concentration step, diafiltration against the loading buffer for the next purification step was also done.

Ion exchange chromatography (IEC). The UF3 protein fraction was loaded onto a 5 ml prepacked column (Pharmacia HiTrap SP) and washed with 25 ml of loading buffer (50 mM Na-acetate buffer, pH 5.0) at a flow rate of 2 ml min⁻¹. Bound proteins were eluted from the column with a biphasic gradient of NaCl in loading buffer that was linear from 0 to 0.53 M in 75 ml (7 mmol ml⁻¹) and from 0.53 to 1.0 M in 1 ml, followed by washing with 25 ml of elution buffer. Protein was monitored at A₂₈₀; fractions of 2.5 ml were collected.

Hydrophobic interaction chromatography (HIC). Fractions from the IEC column containing the majority of xylanase activity were pooled to give the IEC3 fraction (Fig. 4a and b). This fraction was made 1.3 M by slow addition of finely ground (NH₄)₂SO₄. The resul-

tant solution was loaded in 2 ml aliquots and at a flow rate of 0.2 ml min⁻¹ onto a 1 ml prepacked column (Pharmacia Resource PHE) equilibrated with 1.5 M (NH₄)₂SO₄ in 50 mM Na-acetate buffer, pH 5.0 and connected with an HPLC system. The column was washed with 20 ml of equilibration buffer at a flow rate of 1 ml min⁻¹, and bound proteins were eluted with a linear gradient of 1.5 to 0 M (NH₄)₂SO₄ in Na-acetate buffer (75 mmol ml⁻¹). Fractions (1 ml) containing xylanase activity were dialysed against water onto Pharmacia PD10 columns, lyophilised (SpeedVac) and stored at -20°C for several months without showing appreciable loss of activity. EIX was obtained in a highly purified form from *T. viride* liquid cultures according to Dean and Anderson (1991).

Preparative native-PAGE. Native acidic PAGE was performed by modifying the method of Boulikas (1985) in order to have 500 mM acetic acid, 200 mM glycine in the tank buffer and 125 mM Tris-acetic acid, pH 6.4 in the gel (1.5 mm thick). Active bands were detected by the RBB-overlay technique and excised from the gel. Proteins were extracted by freeze-thaw cycles followed by grinding in the buffer needed for the following analyses.

Analysis of eliciting activity. Samples to be tested were applied to tomato (cv. 'Bonny Best') leaflets by the hanging drop method as described by Dean and Anderson (1991). After absorption of the droplet, leaflets were transferred to moist chambers at room temp. Cell suspensions were obtained from calluses of the same tomato cultivar as described in Mozzetti *et al.* (1995) and kept on MS medium (Murashige and Skooge, 1962) supplemented with 1 mg l⁻¹ NAA, 1 mg l⁻¹ 2,4-D and 0.1 mg l⁻¹ kinetin. The cell suspensions were maintained on a rotary shaker (100 rpm) and transferred to fresh medium every week. Three- to four-day old cell suspensions were collected by centrifugation (180 g x 5 min), resuspended in 175 mM mannitol, 500 nM CaCl₂, 500 nM K₂SO₄ and 2 mM Mes-Tris, pH 5.75 (Mozzetti *et al.*, 1997) and placed on a rotary shaker (100 rpm) for 15 min. Samples to be tested were added to a 5 ml cell suspension in a final volume of 100 µl, and changes of conductivity and pH were immediately and continuously monitored for up to 1 h (Orion 101 Conductivity Meter and Sentron 2001 pH System). Cell suspensions were slightly stirred and kept at room temperature in the light during the experiments. Cell viability was checked by the FDA method (Widholm, 1972) by counting at least 200 cells/smear.

RESULTS

Enzyme production. *Fol* secreted xylanase when grown on modified mineral medium containing cell walls, commercial or soluble xylan as a carbon source. Lower activities were detected in the presence of pectin, polygalacturonic acid, carboxymethylcellulose, pectic acid and simple sugars (Fig. 1a). The initial culture pH also affected xylanase production. Changing the pH of the medium from 4.0 to 9.0 before culturing resulted in a 7.5-fold increase in total xylanase activity, the bulk of this increase occurring between pH 5.5 and 6.5. During fungal growth the culture pH shifted towards neutrality (Fig. 1b). Maximal levels of activity were already detected after 24 h of incubation, even though fungal growth would continue for at least 7 more days (Fig. 2a). When a *Fol* culture grown for 3 days in a non-inducing medium of 0.1% D-glucose was shifted to 0.1% soluble xylan, significant levels of xylanase were apparent 1 h after xylan addition and total activity levelled off at 3-4 h (Fig. 2b). For the production of xylanase for purification, modified mineral medium plus 0.1% soluble xylan, pH 7.5 was retained. *Fol* cultures were harvested after 3-4 days of incubation on a rotary shaker (100 rpm).

Enzyme purification. Four xylanases were resolved by a combination of ultrafiltration (UF), cation-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC) and preparative-PAGE. They will be referred to as xylanases i-1, i-2, i-3 and i-4, ranked by decreasing molecular mass (Fig. 3a). Purification was followed by SDS-PAGE and silver staining (Fig. 3b). Xylanase activity was initially purified by UF, followed by IEC. This allowed the purification of i-4 and the recovery of most of the xylanase activity in the IEC3 peak (Fig. 4a). This same peak was fractionated by HIC. As determined with the reducing group assay, two peaks of activity were eluted, corresponding to i-1 and i-3 (Fig. 4b). A second run of the pooled fractions corresponding to i-1 allowed a better purification of this xylanase. The separation of i-2 was accomplished by native acidic PAGE of IEC3 followed by elution of proteins from active bands and confirmation of the activity and pI of the eluted band by RBB-xylan overlay. Although i-1 and i-3 contained in the IEC3 fraction could be identified in this separation step, the liquid chromatography techniques described were found more suitable to their isolation in fair amounts (data not shown).

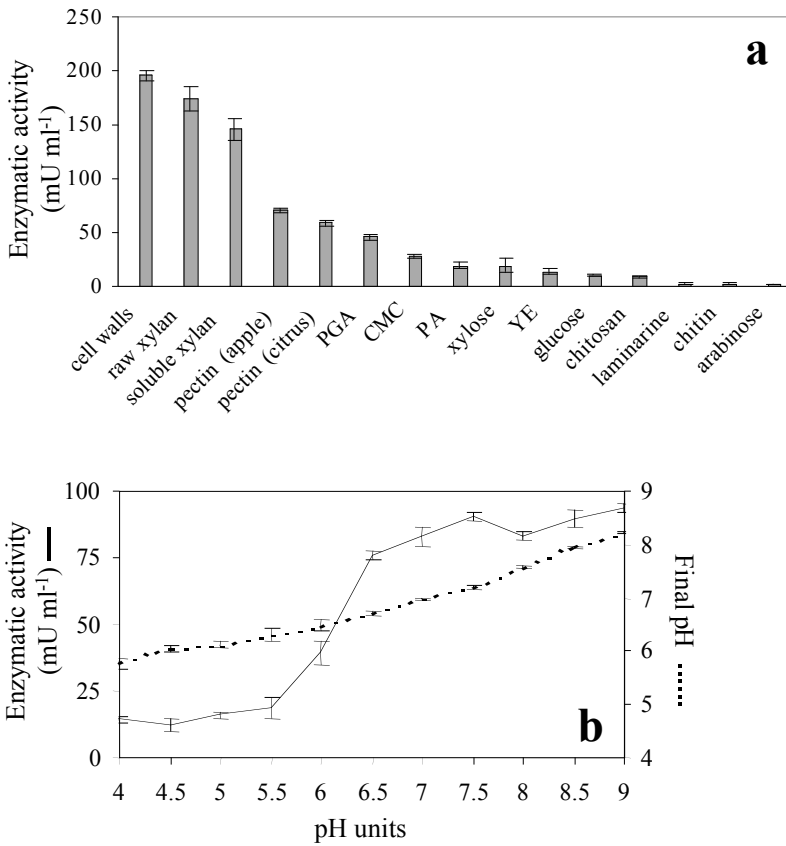


Fig. 1. Inducibility of *Fol* xylanase activity. **a.** Effect of various carbon sources (0.1% w/v) on the induction of *Fol* xylanase activity. All substrates were commercially available, except for the cell wall preparation (obtained as in Ferraris *et al.*, 1996) and the soluble fraction of commercial xylan (obtained as in Doux-Gayat and Auriol, 1976). Four-day old cultures grown on the different substrates were harvested and xylanase activity assessed spectrophotometrically. PGA: polygalacturonic acid; CMC: carboxymethylcellulose; PA: pectic acid; YE: yeast extract. **b.** Effect of initial culture pH on the inducibility of xylanase activity by soluble xylan. Total secreted enzymatic activity was determined in four-day old cultures grown on 0.1% soluble xylan. The initial pH of the medium is given on the x-axis; the pH at harvest is shown as a dotted line.

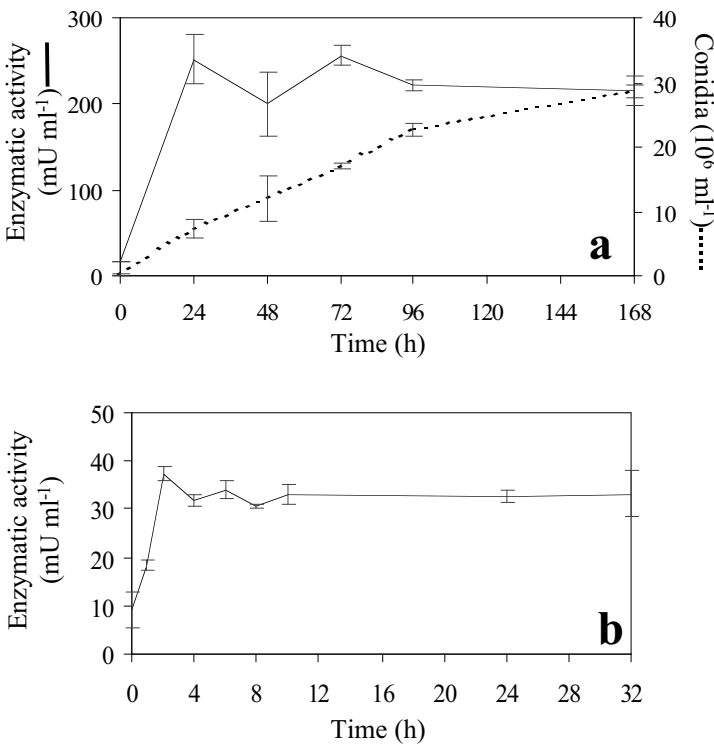


Fig. 2. Time course of *Fol* xylanase induction by soluble xylan. **a.** The fungus was grown directly on 0.1% soluble xylan; aliquots were harvested at different times after the inoculation, between 1 and 7 days. Fungal growth (millions of conidia per ml) is shown as a dotted line. **b.** A 3 day old culture of *Fol* grown on 0.1% D-glucose was supplemented with 0.1% xylan, and the culture was then sampled at intervals for xylanase activity assays.

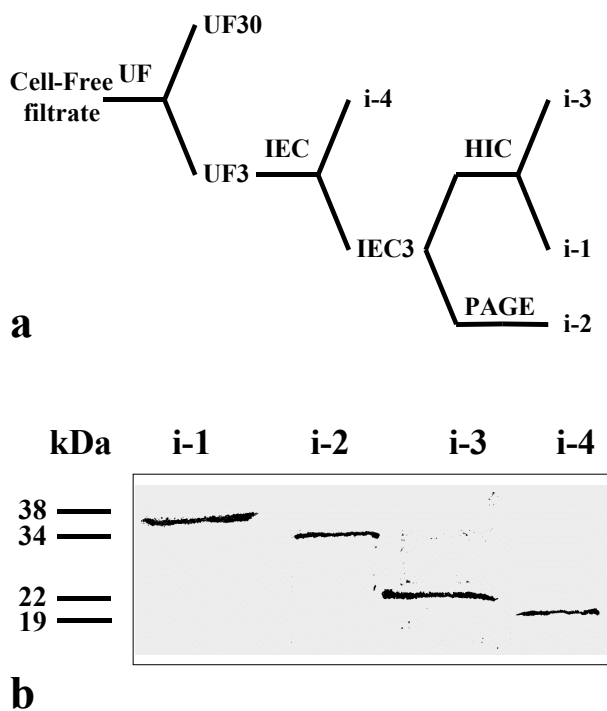


Fig. 3. Purification of *Fol* xylanases. **a.** Purification scheme. CF: Cell-Free filtrate; UF: tangential flow UltraFiltration; IEC: Ionic Exchange Chromatography; HIC: Hydrophobic Interaction Chromatography; PAGE: Polyacrilamide Gel Electrophoresis. **b.** SDS-PAGE analysis of the purified *Fol* xylanases. Protein amounts corresponding to equal xylanase activity in the spectrophotometric assay were separated by SDS-PAGE and silver stained. On the left, sizes in kDa. All four enzymes had a pI > 9.3.

Enzyme characterisation. The molecular masses of the four enzymes were 38, 34, 22 and 19 kDa, respectively (Fig. 3b). Their activity was maximal at pH 6.0 and 45°C (Fig. 5a and b). They all had pI higher than 9.3 and cleaved xylan in an *endo* manner, as shown by the chromatographic analysis of the products of exhaustive xylan digestion by the partially purified xylanase preparation (data not shown). The same analysis conducted on the reaction products of purified i-1 and i-3 showed differences in the cleavage mechanism, revealed by the different elution profiles of digestion products (Fig. 6a, b and c). None of the purified xylanases reacted with the anti-EIX antibodies, and no immunodecorated bands were detected in the crude culture filtrates even when showing very high enzymatic activity (Fig. 7).

Xylanase eliciting activity. As far as the elicitation of physiological responses, negative results were obtained in the cell culture and whole leaf assays for crude fil-

trates and partially purified xylanases from *Fol*. In fact none of the enzyme preparations provoked cell death, pH or conductivity changes upon addition to suspension-cultured cells (up to 5 mU ml⁻¹ final dilution; data not shown), nor to necrotic response on tomato leaflets. The outcome of the treatment by the positive control EIX was comparable with published results, with extended tissue necrosis 6-24 h after infiltration with 5 µg ml⁻¹ EIX and a change in extracellular pH of up to 0.3 units in suspension-cultured cells to which EIX to the same final concentration was delivered (data not shown; Bailey *et al.*, 1990, 1992). Extensive cell death (80% drop in viability 24 h after treatment as compared to controls) could also be observed in EIX-treated suspension-cultured cells but was not induced in samples treated with *Fol* xylanases.

DISCUSSION

We report the purification and partial characterisation of four xylanases produced *in vitro* by *F. oxysporum* f.sp. *lycopersici* (*Fol*). All four enzymes were isolated from *Fol* culture filtrates amended with the soluble fraction of a commercial birchwood xylan preparation. This carbon source represents the best compromise between inducing ability, selectivity in the type of lytic activity induced (cell walls were very good inducers but of a whole range of different enzymes) and easiness of handling (xylan is partly insoluble, and its residues interfere with activity analyses and purification procedures). The inducibility of xylanase activity is confirmed by the fact that the initial culture pH had a significant influence on the ability of *Fol* to secrete xylanases. This is in agreement with results on the *T. viride* EIX (Dean *et al.*, 1989), although with *Fol* there is no sharp peak in productivity at pH 8.0 but a plateau already reached at pH 6.5. The time-course of secretion, also by glucose-established cultures, seems to indicate very rapid induction. Furthermore, xylanase activity in the culture filtrates seems to be poorly related to fungal growth as evaluated by counting the number of conidia, and to be almost completely dependent on the presence of the inducing substrate.

The purification of the four enzymes was checked by SDS-PAGE followed by silver staining; they have molecular masses of 38, 34, 22 and 19 kDa and a pI higher than 9.3. Temperature and pH *optima* are compatible with values reported for other xylanases (see for ex. Dean and Anderson, 1991; Monti *et al.*, 1991; Baker *et al.*, 1992; Holden and Walton, 1992), though with slight differences. The purification procedure, combining ion exchange (IEC), hydrophobic interaction chro-

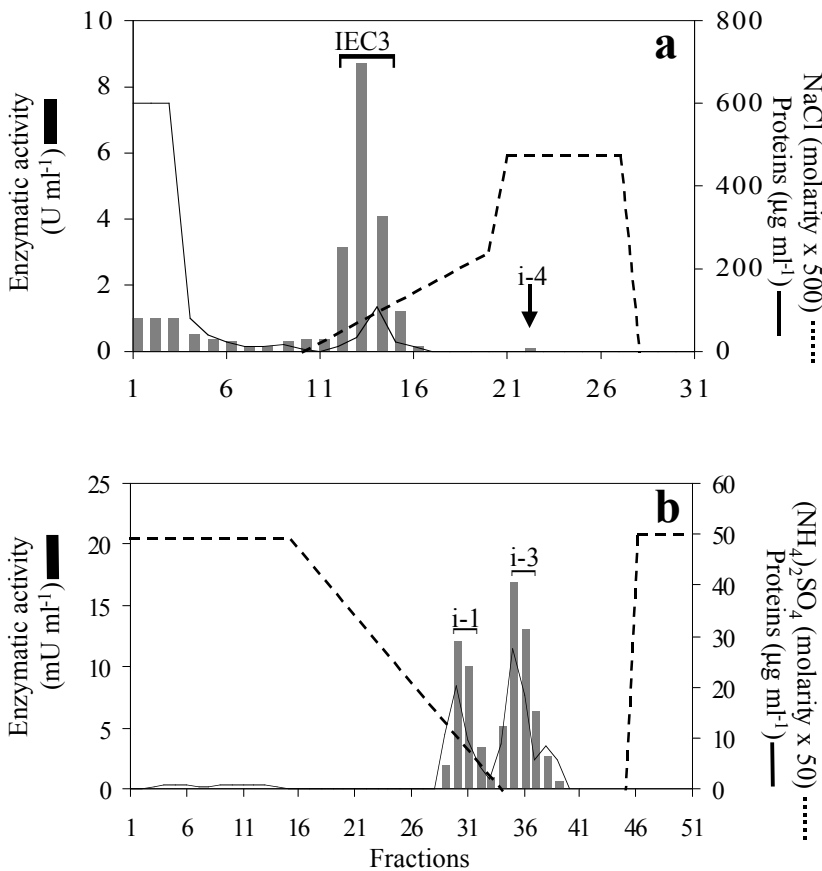


Fig. 4. Xylanase purification by liquid chromatography. **a.** Cation exchange chromatography of 25 ml of the UF fraction enriched in proteins of molecular mass in the range 3-30 kDa (UF3, Fig. 4a) led to the purification of i-4 and the identification of the IEC3 peak as containing most of the enzymatic activity of the cell-free filtrate. Nelson reactions were run on 100 µl of suitably diluted fractions and referred to their original volume (2.5 ml). The i-4 fraction is indicated by an arrow; i-4 absolute activity values are too low compared to those of IEC3 to be clearly shown on the same graph. **b.** The most active fractions of the IEC3 peak were pooled and 2 ml loaded onto a hydrophobic interaction chromatography column, leading to the resolution of i-1 and i-3. One-ml fractions were collected and enzymatic activity assessed spectrophotometrically on soluble xylan.

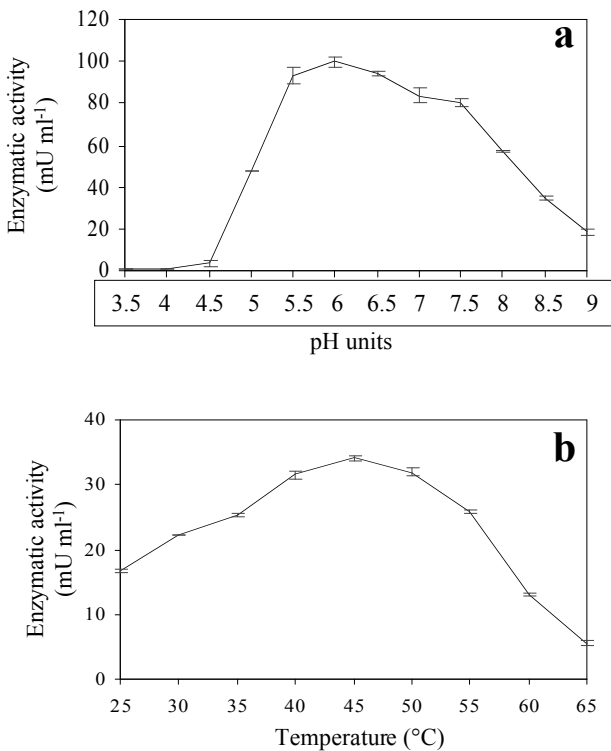


Fig. 5. pH and temperature dependence of partially purified *Fol* xylanase activity. **a.** Influence of pH on xylanase activity was evaluated in the range 3.5 to 9. Suitably diluted aliquots of the IEC3 peak were assayed by the Nelson test for the release of reducing sugars. The pH of the test buffer (50 mM Na acetate) was adjusted to the values indicated on the x-axis with either acetic acid or NaOH. **b.** Temperature sensitivity of *Fol* xylanases was assayed by a standard 10-min activity test in the range of 25 to 65°C. Sugars released were quantified by the Nelson method.

matography (HIC) and preparative native-PAGE electrophoresis, focussed on low-molecular weight, basic xylanases such as EIX. Recently, Christakopoulos *et al.* (1996) purified a major xylanase with cellulase and transferase activities from *F. oxysporum* strain F3. This enzyme has a molecular mass of 60.2 kDa and a pI of 6.6; it is an *endo*-xylanase, which has low cellulase and transferase activities and constitutes around 75% of the total secreted protein. The different fungal strain may account for the fact that in our case most of the activity seems to be due to high-pI isoforms. This was confirmed by the IEF electrophoretic pattern of active

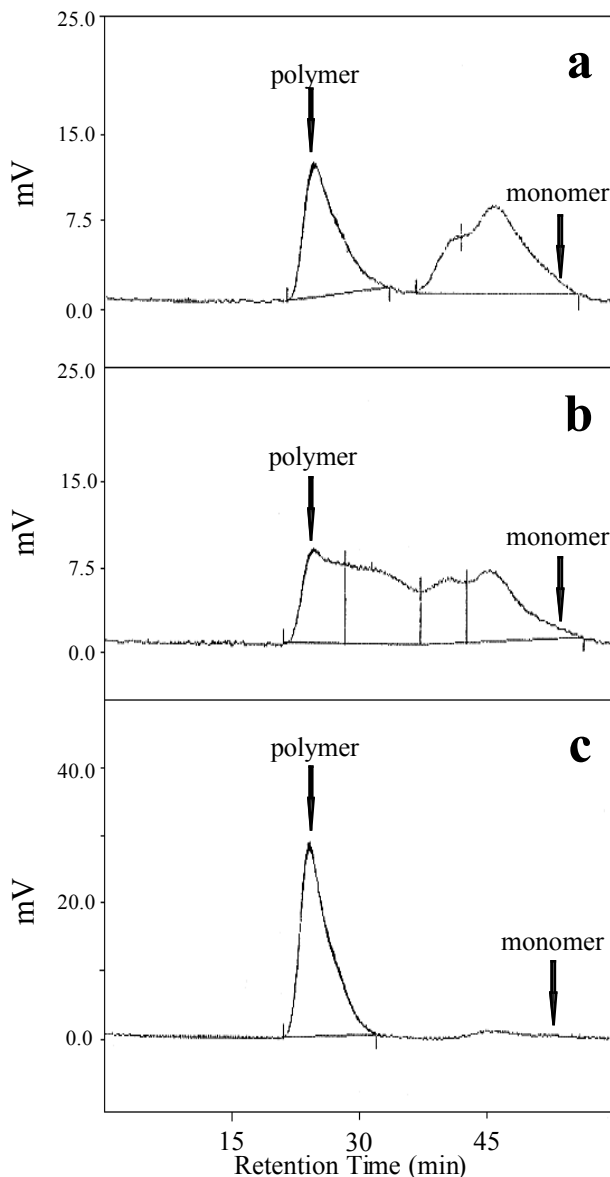


Fig. 6. Characterisation of the cleavage mechanism of i-1 and i-3 by size exclusion chromatography. Oligosaccharides released from 0.3% soluble xylan after 1 h incubation at 40°C in the presence of comparable amounts of (a) i-1 and (b) i-3 or buffer (c) were fractionated by gel filtration chromatography on Sephadex G-25. The column (1.6 x 40 cm) was calibrated with Blue dextran (V0), low mol. wt malto-oligosaccharides (D.P.=10-4; Pfanstiehl Laboratories, Inc., Waukegan, IL), raffinose (D.P.=3), saccharose (D.P.=2) and xylose (D.P.=1). The elution of the reaction products was continuously monitored by refractometry, and is reported here as elution profiles in mVolts (mV).

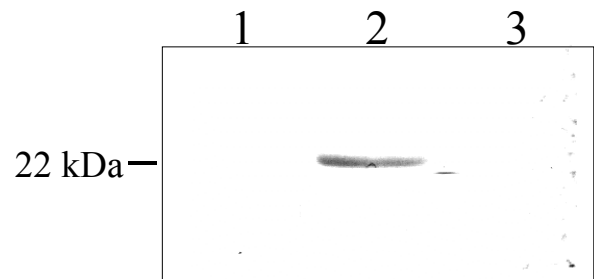


Fig. 7. Immunoblot analysis of *Fol* cell-free filtrates. Samples of xylan-induced *Fol* culture filtrates (corresponding to about 0.46 and 2.3 U) were concentrated 500 and 2500-fold by freeze-drying and dialysis, and then subjected to SDS-PAGE (lane 1 and 3, respectively). 50 ng of pure EIX were loaded as positive control on lane 2. After protein transfer to nitrocellulose membranes, crude anti-EIX antiserum was used as a primary antibody at a 1:5000 dilution in blocking buffer (Johnson *et al.*, 1984).

bands in crude filtrates revealed by the RBB-xylan overlay technique (data not shown) and by the high percentage of activity retained on IEC columns. The purification protocols take advantage of this feature.

Overall purification of the four xylanases gave a very low yield, and the calculated specific activity (Table 1) does not reflect the true purification rate. As previously observed during the purification of other xylanases, several factors probably contributed to this apparently low yield. Commercial preparations of xylan are normally not pure and contain small percentages of other sugars (see for example Joselau *et al.*, 1992). Any enzyme present in *Fol* culture filtrates and capable of releasing such sugars would in the early stages of purification contribute to apparent xylanase activity in the Nelson test. Furthermore, salts [especially $(\text{NH}_4)_2\text{SO}_4$] in column fractions interfere with this test, also affecting the enzymatic activity and modifying the quantitative results. In addition, a synergistic effect is likely to take place when different isoforms of the same enzyme are able to cooperate in the depolymerisation of the substrate (see for example Wong *et al.*, 1988; He *et al.*, 1994); this is of course less and less probable as purification proceeds. This may account in our view for the apparent loss in specific activity shown in Table 1.

Chromatographic analyses of the products of exhaustive xylan digestion by the partially purified xylanase preparations showed that all four enzymes prevalently cleave xylan in an *endo*-manner. Nevertheless, analysis of the reaction products of i-1 and i-3-enriched fractions shows differences in the cleavage mechanism, which is likely to be less random (*i.e.* closer to an *exo*-mechanism) for i-1 than for i-3. The production of

Table 1. Purification of basic *endo*-xylanases from *Fol* cell-free culture supernatant.

Purification step	Volume ml	Total protein mg	Enzymatic activity U	Specific activity U mg ⁻¹	Yield %	Enrichment-fold
Cell-free filtrate	2000	68.800	291.14	4.23	100.000	1.00
Ultra filtration	50	21.310	168.18	7.90	57.800	1.86
Ionic exchange chrom.	20	0.880	17.44	19.82	5.990	4.69
Hydrophobic interaction chrom. (i-1)	20	0.300	0.22 ^a	0.73 ^a	0.076 ^a	0.17 ^a
PAGE (i-2)	50 µl	n.q. ^b	n.q. ^b	–	–	–
Hydrophobic interaction chrom. (i-3)	20	0.460	0.30 ^a	0.65 ^a	0.100 ^a	0.15 ^a
Ionic exchange chrom. (i-4)	5	0.023	0.21	9.13	0.070	2.16

^a See text for discussion of results; salts interfered with enzyme and protein assays.

^b Non quantifiable. Recovered protein merely allowed detection on IEF-PAGE zymograms and SDS-PAGE; spectrophotometric assays could not therefore be performed.

multiple xylanases has been reported for a wide variety of xylanolytic microorganisms (Wong *et al.*, 1988; Wu *et al.*, 1997). Monti *et al.* (1991) reported that the two main xylanases from *Humicola grisea* differ in the dynamics of substrate demolition, even though both are *endo*-enzymes. The different isozymes are expected to differ in their specificity (Elegir *et al.*, 1994) and to have a synergistic effect on the process of xylan hydrolysis (He *et al.*, 1994); the same could be true for the i-1 and i-3 enzymes from *Fol* purified in this study.

A role in pathogenesis was proposed in the past for EIX and EIX-like enzymes, since antigenically related proteins had been found in the culture filtrates of several phytopathogenic fungi, among which *F. oxysporum* f.sp. *pisi* (Dean *et al.*, 1989). Furthermore, sensitivity to EIX had been shown to be controlled by a single, dominant gene both in tobacco and in tomato plants (Bailey *et al.*, 1993; Avni *et al.*, 1994a). It was therefore suggested that this and related molecules may be involved in a wide range of plant-pathogen interactions, acting as proteinaceous (non-enzymatic) elicitors of active defence mechanisms (Dean *et al.*, 1989; Walton, 1994). We therefore decided to preliminarily test the possible recognition of *endo*-xylanases from *Fol* by the host plant, testing them for EIX-like eliciting activities. In our work, *Fol* proved to secrete abundant xylanase activity upon induction by xylan; some of the enzymes responsible for such activity were purified and partially characterised. Although sharing several features with the *T. viride* EIX (function, low mol. wt and high pI, pH and temperature *optima*, mode of induction), they were not antigenically related to it. Furthermore, neither the crude cell-free culture filtrates nor the purified xylanases showed any EIX-like eliciting activity on in-

tact tomato leaflets or cell suspensions. The lack of appreciable cell death- or electrolyte leakage-inducing activities in our experimental conditions as well as the antigenic differences from EIX suggest that a role as recognition factors for these enzymes is unlikely, both when their enzymatic activity and primary structure are considered. More detailed analyses would be required to exclude any biological, non-enzymatic activity in plant tissues of xylanases other than EIX such as the enzymes isolated from *Fol*. Nevertheless, evidence is accumulating that the enzymatic activity of xylanases, and among them EIX, is not involved in elicitation, as suggested by Fuchs *et al.* (1989) and shown by Enkerli *et al.* (1999), Sharon *et al.* (1993) and Yano *et al.* (1998). In addition, the results reported by Yano *et al.* (1998) together with ours seem to define EIX as a more isolated case among xylanases produced by different organisms than previously thought (Dean *et al.*, 1989; Walton, 1994). This protein would act as an elicitor of cell death upon recognition of specific, yet unidentified structural features by sensitive tissues. Such features would not be shared by other known proteins even if structurally and/or functionally related (Yano *et al.*, 1998).

Different sets of cell-wall degrading enzymes are frequently expressed in different culture conditions. This may reflect what happens in nature during pathogenic and saprophytic growth, and it is illustrated by the results of experiments of xylanase genes knockout. Wu *et al.* (1997) showed by a gene disruption approach that the rice blast fungus *Magnaporthe grisea* can secrete different sets of xylanases (at least six different ones) probably according to environmental and growth conditions. These differences are likely to confer more flexi-

bility on the microorganism in the degradation of naturally complex substrates as arabinoxylans (Wu *et al.*, 1997, and references therein); the multiplicity of enzymatic forms normally observed does not mean functional redundancy. For this reason, caution should be exerted in interpreting the results obtained with enzymes produced *in vitro*.

ACKNOWLEDGMENTS

The National Research Council of Italy (Special Project RAISA, Subproject no. 2) supported this work. The authors are grateful to Dr. J. D. Anderson (ARS-USDA, Beltsville, MD, USA) for the kind gift of anti-EIX antibodies and for critical revision of the manuscript.

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Received 21 March 2000

Accepted 16 October 2000