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Extracellular xylanases from two pathogenic races of *Fusarium oxysporum* f. sp. *ciceris*: enzyme production in culture and purification and characterization of a major isoform as an alkaline endo-β-(1,4)-xylanase of low molecular weight

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Abstract

Fusarium oxysporum f. sp. *ciceris*, the causal agent of Fusarium wilt of chickpea, comprises eight pathogenic races and two pathotypes. Races 0 and 5, representative of the least virulent yellowing pathotype and the most virulent wilt pathotype, respectively, produced extracellular xylanases when grown on minimal medium supplemented with either 1% commercial birchwood xylan or 0.3% chickpea cell walls. The pattern of extracellular proteins analysed by denaturing polyacrylamide gel electrophoresis in the two media presented some minor but distinctive differences between fungal races. By preparative isoelectrofocusing, the xylanase activity in cell wall-culture filtrates could be resolved into basic and neutral fractions with p*I* values around to 10 and 8, respectively, whereas the xylan-culture filtrates contained an additional acidic fraction of p*I* around 4. A common major xylanase was purified 7-fold to homogeneity by cation-exchange chromatography and chromatofocusing. The purified xylanase has a molecular weight of 21.6 kDa, optimum pH and temperature of 5.5 and 55 °C, respectively, p*I* in the range of 8.2 to 9.0, and K_m and V_{max} values of 2.24 mg ml^{-1} (birchwood xylan as substrate) and 1200 nkat mg^{-1} protein (72 U mg^{-1} protein), respectively. The enzyme has an endo mode of action, hydrolysing xylan to xylobiose and higher short-chain xylooligosaccharides without forming free xylose.

Abbreviations: SDS-PAGE – denaturing polyacrylamide gel electrophoresis; IEF – isoelectrofocusing; U – International unit of enzyme activity (μmol min^{-1})

Introduction

Xylan, the main constituent of plant cell wall hemicelluloses, is a heterogeneous polymer consisting primarily of a linear β-(1,4)-D-xylan backbone partially acetylated and substituted with a variety of side chains, mainly including single α-D-glucuronosyl (and its 4-O-methyl ether) and α-L-arabinosyl units. The structural complexity of xylan suggests that several hydrolases must be required for its complete degradation; however, the key enzyme is xylanase or, more properly, endo-β-(1,4)-xylanase (EC 3.2.1.8). That enzyme cleaves the internal β-(1,4) bonds in the xylan backbone at nonmodified residues, yielding different chain length substituted xylooligosacharides. Recently, the structural and functional properties of several xylanases have been studied in detail with the majority of known microbial xylanases being included into families 10 (formerly F) and 11 (formerly G) of glycosyl hydrolases (Biely et al. 1997; Kulkarni et al. 1999; Sapag et al. 2002). In addition to these basic enzymological studies, microbial xylanases have received considerable attention from two more applied fields, e.g. biotechnology and plant pathology. Whereas biotechnology-oriented studies have tended to emphasize the potential industrial uses of xylanases (e.g. in pulp and paper, bioconversion, feed and food industries (Kulkarni et al. 1999)), phytophatology-oriented studies have addressed mainly the ability of xylanases to degrade plant cell walls (Walton 1994) and, in certain instances, for inducing plant defence responses (Dean et al. 1989; Lotan and Fluhr 1990).

Fusarium oxysporum is an ubiquitous, soilborne fungal species complex comprised of saprophytic and pathogenic isolates. Pathogenic isolates of *F. oxysporum* are assigned to *formae speciales* based on host plant specificity and further into pathogenic races based on host cultivar specificity (Gordon and Martyn 1997). About 80 *formae speciales* have been identified, most of which cause severe wilt diseases to many economically important crops. Phytopathogenic forms of *F. oxysporum* enter roots of host plants and grow through the cortex into the xylem vessels where they impair the plant water-conducing ability and produce wilting. Plant cell wall degradation may be crucial for pathogenesis in wilt diseases since it must be required initially for pathogen ingress into the host xylem and thereafter for fungal nutrition within the xylem. Consequently, plant cell wall degradation by extracellular fungal enzymes may play a primary role in different phases of wilt diseases. Previous studies reported purification and/or characterization of xylanases from *F. oxysporum* f. sp. *melonis* (Alconada and Martínez 1994) and *F. oxysporum* f. sp. *lycopersici* (Ruiz et al. 1997; Ruiz-Roldán et al. 1999; Cardinale and Matta 2001; Gómez-Gómez et al. 2001, 2002), as well as from the presumably nonpathogenic *F. oxysporum* F3 strain isolated from cumin (Christakopoulos et al. 1996a, b; 1997). Results from those studies clearly indicated that differences in the major *in vitro*-expressed xylanase forms can occur when comparing *F. oxysporum formae speciales* or strains, or even different isolates of a given *forma specialis* or strain. However, no studies had been carried out so far comparing races of a *F. oxysporum* f. sp.

Fusarium oxysporum f. sp. *ciceris* (Foc), the causal agent of Fusarium wilt of chickpea (*Cicer arietinum* L.), exhibits considerable pathogenic variation even though it is monophyletic (Jiménez-Gasco et al. 2002). Two pathotypes, namely yellowing and wilting, and eight races (races 0, 1A, 1B/C, 2, 3, 4, 5, and 6) have been differentiated by pathogenicity tests (Haware and Nene 1982; Jiménez-Díaz et al. 1994) and polymerase-chain-reaction assays (Kelly et al. 1994; Jiménez-Gasco et al. 2001; Jiménez-Gasco and Jiménez-Díaz, 2003). Race 0 is the least virulent of all races and induces foliar yellowing while race 5 is the most virulent race and induces severe wilt (Jiménez-Díaz et al. 1994; Navas-Cortés et al. 2000). In previous work we purified and characterized the main pectic enzymes produced *in vitro* by Foc, and showed that races 0 and 5 differ in their enzyme production (Pérez-Artés and Tena 1990). As an extension of that study, in the present work we have compared Foc races 0 and 5 for *in vitro* xylanase production. In contrast to the pectic enzymes, less remarkable differences were found between races 0 and 5 with respect to their xylanase complements as the two races produce the same major xylanase form, the purification and characterization of which is also described in the present work.

Materials and methods

Fungal isolates and cultures

Monoconidial *F. oxysporum* f. sp. *ciceris* isolates Foc 7802 and Foc 8012, representative of Foc races 0 and 5, respectively, were used. These isolates were obtained from infected chickpea in southern Spain and used in previous studies (Pérez-Artés and Tena 1989, 1990; Jiménez-Gasco et al. 2001). Cultures of isolates were stored in sterile soil in test tubes at 4 °C. Active cultures of isolates were obtained from small aliquots of the soil culture on potato-dextrose agar (Trapero-Casas and Jiménez-Díaz 1985). Inocula and liquid cultures were prepared as previously described (Pérez-Artés and Tena 1989), except that liquid cultures contained 1% (w/v) birchwood xylan (Sigma, St. Louis, MO, USA) instead of pectin as carbon source and were incubated at 14-h photoperiod of fluorescent light (360 μE $m^{-2}s^{-1}$) rather than in darkness. Mycelia from cultures were separated by centrifugation (15000*g*, 30 min, 4 °C) and the supernatant was filtered through a 0.22 μm membrane (Millipore, Madrid, Spain), dialyzed against deionised water and used as crude enzyme preparation.

Spectrophotometric xylanase and protein assays

Xylanase activity was assayed by following the release of reducing groups from commercial birchwood xylan as substrate, using the Somogyi-Nelson method with D-xylose as standard (ε_m = 5518 M^{-1} cm^{-1} at 520 nm) (Pérez-Artés and Tena 1989). The reaction mixtures, containing 400 μl of 0.1% (w/v) xylan in 50 mM sodium acetate buffer (pH 4.5) and 100 μl enzyme, were incubated at 37 °C for 30 min. Similar mixtures but containing boiled enzyme were included as blanks. Enzyme activity was expressed in nkat (nmol s^{-1}). Protein in enzyme preparations was determined by the method of Bradford (1976), using bovine serum albumin as a standard.

Xylanase fractionation and purification

The profile of xylanases secreted by Foc races 0 and 5 was obtained by preparative isoelectrofocusing (IEF) of dialyzed filtrates from highest activity cultures on a Rotofor System (BioRad, Richmond, California, USA). The IEF run was carried out with 55 ml culture filtrate containing 2% (w/v) carrier ampholytes (Bio-Lyte 3–10; BioRad) at 4 °C and 15 W constant power for 2.5 h. Twenty fractions were collected and the pH and xylanase activity of each fraction were determined.

Xylanase was purified by a combination of cation exchange chromatography (CEC) and chromatofocusing (CF). Dialyzed filtrates from cultures of highest xylanase activity were concentrated by freeze-drying and redissolution in a small volume of 50 mM sodium acetate buffer pH 4.5 (typically, 200 ml of dialyzed culture filtrates were redissolved in 25 ml of acetate buffer). CEC was performed using a mono S HR 5/5 column coupled to a FPLC System (Pharmacia Biotech, Uppsala, Sweden). Two ml of concentrated enzyme were loaded onto the column, which had been previously equilibrated with 50 mM sodium acetate (pH 4.5), then that was washed with 6 ml equilibrating buffer, and the bound proteins were eluted with a linear 0 to 0.5 M gradient of NaCl in the above buffer at a flow rate of 1 ml min^{-1}. Fractions of 1 ml were collected and assayed for xylanase activity. Fractions containing the main peak of several CEC runs were combined to obtain sufficient amount of purified xylanase. Those combined fractions were dialysed and then subjected to FPLC CF on a mono P HR 5/5 column (Pharmacia Biotech) equilibrated with 25 mM diethanolamine-HCl buffer (pH 9.5). Proteins were eluted with 7 ml equilibrating buffer followed by 13 ml of 1:10 diluted Polybuffer 96 (Sigma)-acetic acid (pH 6.0) at a flow rate of 0.8 ml min^{-1}. Fractions of 1 ml were collected and their pH and xylanase activity were determined.

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE. Extracellular proteins were subjected to PAGE under denaturing conditions on 3% stacking and 12% resolving gels in a Mini-Protean II (BioRad), according to Laemmli (1970). The resolved protein bands were visualized after staining with Coomassie Brilliant Blue R (Sigma), and their molecular weights (MWs)

were calculated using SDS-MW markers (Sigma).

IEF-PAGE. Isoelectrofocusing was performed using 0.4 mm polyacrylamide gels which were cast on a horizontal slab unit (Model 111 Mini, Bio-Rad) according to manufacturer's instructions. Salt-free enzyme samples were applied and focused at constant potential of 100 V for 15 min, followed by 200 V for 15 min and 450 V for 60 min at room temperature. Isoelectric points were estimated using IEF protein standards (BioRad) with p*I* ranging from 4.45 to 9.6.

Zymogram. For zymogram analysis, enzyme samples were subjected to native PAGE. Electrophoresis was performed cathodally according to Reisfeld et al. (1962) with 7.5% stacking and 15% resolving gels, at a constant current of 35 mA for 4 h. About 2 μg of protein were applied to each gel lane. Following electrophoretic separation, xylanase activity was detected by an overlay technique (Royer and Nakas 1990). Briefly, the polyacrylamide gel was covered with an ultra thin 1% (w/v) agarose gel (thickness of 1 mm) containing 0.1% (w/v) RBB-xylan (Loewe Biochemica GmbH, Sauerlach, Germany) in 50 mM sodium acetate (pH 4.5) and incubated at 37 °C for 2 h. Activity bands were visualised as clear zones on the deep-blue RBB-xylan background after the dye-coupled xylan fragments produced by enzyme hydrolysis were washed off with 95% (v/v) ethanol.

Mode of action

The products of enzymatic hydrolysis formed in 2% (w/v) xylan reaction mixtures at 37 °C and pH 4.5 were examined by ascending TLC on silicagel 60 precoated plates (Merck, Darmstadt, Germany), using ethyl acetate-acetic acid-water (18:7:8, v/v/v) as mobile phase and alkaline $AgNO_3$ as spray reagent. At defined times, reaction mixtures were sampled, the enzyme activity was stopped by boiling for 10 min, and 4-μl-aliquots of samples were applied on TLC plates. Development was made with alkaline $AgNO_3$ reagent according to Krebs et al. (1969). Briefly, TLC plates were sprayed with a saturated $AgNO_3$ solution in acetone with a minute amount of water and then with 0.5 N aqueous-methanolic NaOH, and finally heated at 100 °C for 1–2 min.

Results

Enzyme production

Xylanase production by races 0 and 5 of *F. oxysporum* f. sp. *ciceris* took place with a similar time course, although it varied depending upon the carbon source used (Figure 1). Thus, xylanase activity with xylan as carbon source was low (Foc race 0) or very low (Foc race 5) at day 3, increased to a maximum level by day 7, and remained close to this value at day 10. In parallel to this enzyme activity pattern, the pH value of culture media increased from near neutral value at day 3 (6.4 and 7.4 for Foc races 5 and 0, respectively) to a constant alkaline value of 8.5 by days 7 and 10 (Figure 1a). Conversely, xylanase activity using chickpea cell walls as carbon source was maximal at day 3 and then declined to low or null values at days 7 and 10, respectively, whereas the culture medium pH remained constant at about 7.0 along the entire sampling period Figure 1b).

Culture filtrates of highest xylanase activity were subjected to SDS-PAGE and preparative IEF analyses to determine whether heterogeneity in their protein and xylanase complements might occur. SDS-PAGE analysis revealed interesting comparisons between the protein bands associated with either carbon source or pathogen race (Figure 2). Seven-day-old Foc races 5 and 0 cultures on xylan as carbon source had two main protein bands with molecular weight values near to 24 and 20 kDa, respectively; although the former was nearly absent in Foc 0 cultures (Figure 2, lanes 1 and 2). In contrast, 3-day old Foc cultures on chickpea cell walls showed four main protein bands, of which the two bands with molecular weights of about 60 and 40 kDa, respectively, were minimally represented in the xylan cultures, if at all. The two remaining main protein bands, in turn, had molecular weights similar to those of the two major protein bands in xylan cultures (Figure 2, lanes 3 and 4). While three of the four main protein bands described above were equally abundant in cultures of both Foc 0 and Foc 5, the band of about 24 kDa was poorly represented in Foc 0 cultures. Assuming that xylanases are likely to be proteins abundantly expressed both in xylan and plant cell wall cultures of Foc races 0 and 5, the 20 kDa band is the most obvious candidate for harbouring the

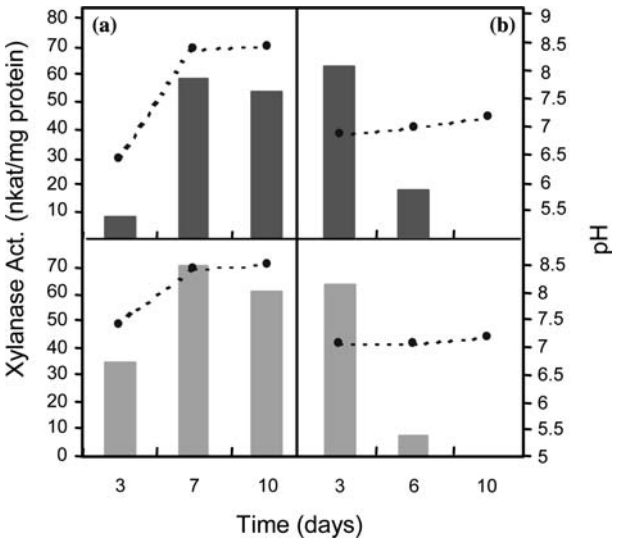

Figure 1. Xylanase activity and pH value of culture filtrates from *Fusarium oxysporum* f. sp. *ciceris* (Foc) races 0 and 5. Filtrates from cultures on 1% (w/v) xylan (a) or 0.3% (w/v) chickpea cell walls (b) were analysed for xylanase activity (■ and ■ for Foc race 5 and Foc race 0, respectively) and pH value (.....).

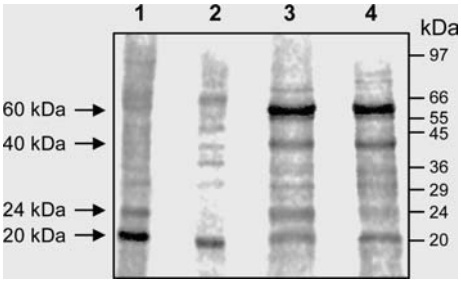

Figure 2. SDS-PAGE of culture filtrates from *Fusarium oxysporum* f. sp. *ciceris* (Foc) races 0 and 5. Lanes 1 and 3 refer to cultures of Foc race 5 on xylan and chickpea cell walls, respectively; lanes 2 and 4 refer to cultures of Foc race 0 on xylan and chickpea cell walls, respectively. Resolved proteins were stained with Coomassie Brilliant Blue R.

main form of such enzyme activity common to both Foc races (see below). In contrast, the band of about 24 kDa, which was scarcely present or even absent in Foc 0 cultures, could be considered a putative xylanase specifically expressed by the most virulent race 5 of Foc.

Preparative IEF analysis of cell wall-culture filtrates at pH of 3 to 10 resolved the xylanase activity into one basic fraction of p*I* of about 9.0 to 10.5 and another neutral one of p*I* around 7.0 to 8.0 (Figure 3b). Conversely, analysis of xylan-culture filtrates revealed a further acidic fraction which p*I* was around 3.0 to 5.0 (Figure 3a).

Xylanase purification

Purification of xylanase from 7-day-old xylan culture filtrates of Foc races 0 and 5 yielded similar results for the two races; therefore, results presented here apply to the xylanase of each of them. Preliminary xylanase purification, including preparative IEF in the pH range 3–10 or gel filtration chromatography on Sephacryl S-200 steps, gave unsatisfactory results. The IEF method caused great loss of enzyme activity that was probably a consequence of both the run conditions themselves as well as the ammonium sulphate treatment and dialysis of active fractions for removing ampholyte from them. Similarly, behaviour of Foc xylanase on Sephacryl S-200 was anomalous since it eluted from the column at an elution volume much higher than the total column volume; this indicated that factors other than gel filtration were involved in the enzyme elution. Furthermore, recovery of xylanase activity from the gel filtration step was very low. The purification procedure finally adopted included a freeze-drying concentration step, followed by cation-exchange and chromatofocusing FPLC steps. The cation-exchange chromatographic step resolved the xylanase activity into a small fraction that was not retained by the column (i.e. eluted in the washings) and a main fraction that was eluted in the salt gradient at 0.12 M NaCl

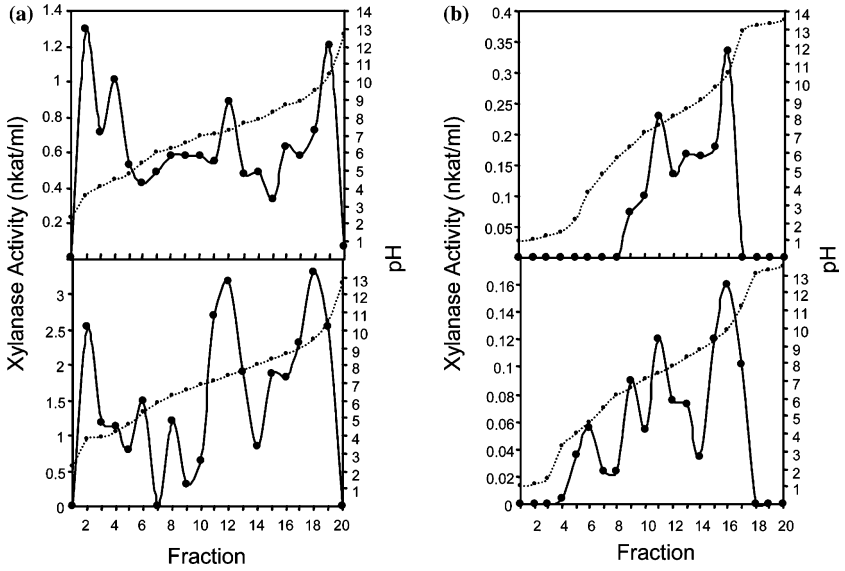

Figure 3. Isoenzyme pattern of xylanase activity in culture filtrates from *Fusarium oxysporum* f. sp. *ciceris* (Foc) races 0 and 5. Filtrates of highest activity cultures on 1% (w/v) xylan (a) or 0.3% (w/v) chickpea cell walls (b) were subjected to preparative isoelectric focusing and the fractions analysed for xylanase activity (top panels and lower panels for Foc race 5 and Foc race 0, respectively) and pH value (.....).

(Figure 4a). This main xylanase fraction was then subjected to a chromatofocusing step at a pH range of 9 to 6 that gave rise to an unique xylanase peak retained very weakly by the column. That peak was eluted in the washings, at the starting (highest) value of the pH gradient (pH 9.0, Figure 4b). Results of the purification procedure referred to 200 ml of culture filtrate are summarised in Table 1. A 16% overall recovery of the activity, with a 7-fold increase in specific activity, was obtained. Comparison of SDS-PAGE runs of samples from the three purification steps indicates that a homogeneous final xylanase preparation was only obtained after the chromatofocusing step (Figure 5a). The purified enzyme also was detected as a single protein band after both IEF-PAGE (Figure 5b) and native-PAGE (Figure 5c) and as a single activity band after zymogram analysis (Figure 5c).

Characterization of the purified xylanase

The purified enzyme showed a molecular weight of about 21.6 kDa as estimated by SDS-PAGE (Figure 5a), a value very close to that of the faster moving major protein band detected in both xylan- and cell wall-cultures of Foc races 0 and 5 (Figure 2). The apparent MW of the native xylanase could not be estimated by gel filtration because, as previously indicated, the anomalous behaviour of the enzyme on Sephacryl S-200 resulted in an elution volume much higher than that corresponding to the lower limit of the MW markers. As microbial xylanases are usually monomeric proteins (Törrönen and Rouvinen 1997), we consider that the SDS-PAGE result provides a good estimation of the purified Foc xylanase MW. After incubation with glycopeptidase F (Sigma), the purified xylanase did not experience any reduction in its MW as estimated by SDS-PAGE (not shown). This result would suggest that, in contrast to the major extracellular xylanase obtained from *F. o. lycopersici* cultures by Ruiz et al. (1997), the Foc enzyme is not a *N*-glycosylated protein. Attempts to obtain the N-terminal sequence of the purified xylanase were unsuccessful, probably due to the presence of a substituted aminoacid at the extreme N-terminus. Xylanases blocked at their N-termini have been previously described (Haas et al. 1992; Holden and Walton 1992) and in some instances a *N*-acetyl group could be identified as the blocking group (Srinivasa et al. 1991).

The p*I* of the purified xylanase was estimated at near to 8.2 by analytical IEF-PAGE (Figure 5b);

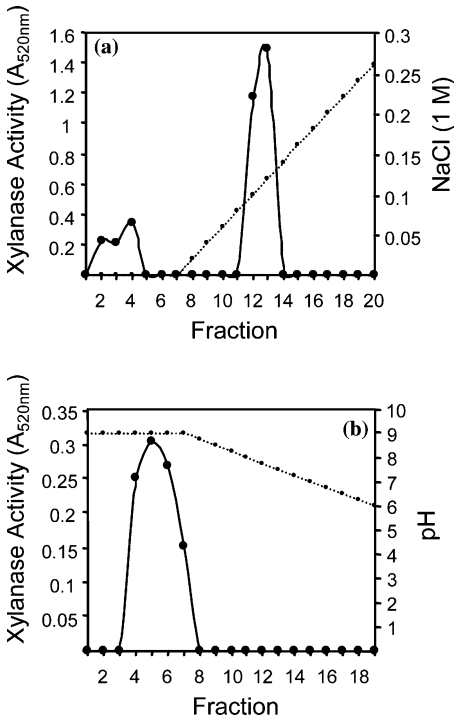

Figure 4. Purification of a major xylanase present in culture filtrates from *Fusarium oxysporum* f. sp. *ciceris* (Foc) races 0 and 5. (a) FPLC cation-exchange chromatography on a mono S H/R 5/5 column of the culture filtrate concentrated by freeze-drying. (b) FPLC chromatofocusing on a mono P H/R 5/5 column of the main xylanase fraction from cation-exchange chromatography step. (●) represents xylanase activity (A_{520nm}) and (.....) represents the gradient of NaCl (a) or pH (b).

however, lack of enzyme fixation to the chromatofocusing column at pH 9.0 (Figure 4b) might indicate a higher value of p*I* (≥9.0). That would be in closer accordance with the results found previously for the basic xylanase fraction in both xylan and cell-wall culture filtrates by means of preparative IEF (Figure 3).

The xylanase purified in this present work had an optimum pH at 5.5, retaining more than 70% optimum activity within the pH range of 4.5 to 6.5. At pH values of 3.5 and 8.5, the extremes of the pH range studied, the enzyme still displayed about 50% relative activities. The optimum temperature for activity of the purified xylanase was 55 °C (range tested, 25–70 °C) and the activation energy for the enzyme reaction calculated from the Arrhenius plot was 16 kJ mol^{-1}. The enzyme retained more than 90% optimum activity within a temperature range of 45–55 °C and showed relative activities higher than 60% between 25 and 40 °C and at 60 °C, whilst the relative activity was lower than 40% beyond 65 °C. The rate dependence of the enzyme reaction on substrate (birchwood xylan) concentration followed Michaelis-Menten kinetics. The apparent K_m and V_{max} values calculated from the Lineweaver–Burk plot were 2.2 mg ml^{-1} and 1200 nkat mg^{-1} protein (72 U mg^{-1} protein), respectively. On oat spelt xylan (Sigma) as substrate, the activity of the purified xylanase was about 40% higher than that on birchwood xylan whereas on carboxymethylcellulose that activity was null.

The mode of substrate hydrolysis by the purified xylanase was studied by ascending TLC analysis of products formed in the enzyme reaction mixture at increasing incubation period up to 48 h. As shown in Figure 6, only xylooligosacaharides decreasing in polymerisation degree up to xylobiose, but no monomeric xylose, were detected. Xylobiose was the main reaction product detected in the 2 to 24 h-reaction mixtures and the only soluble product detected in the 48 h-reaction mixture.

Discussion

In this work, we have studied the ability of *F. oxysporum* f. sp. *ciceris* to produce extracellular xylanases and compared two fungal races (0 and 5) differing in virulence, with respect to such an ability. Previous studies from our laboratory had indicated that *F. o. ciceris* has a high capacity for extracellular production of pectinases but

Table 1. Purification of a major xylanase from xylan culture filtrates of *F. oxysporum* f. sp. *ciceris*.

Purification steps	Total protein (mg)	Total activity (nkat)	Specific activity (nkat mg^{-1} protein)	Purification (fold)	Yield (%)
Crude	11.59	977.1	84.3	1	100
Dialysis and freeze-drying	5.63	517.8	92.1	1.1	53
Mono S	0.82	285.9	348.2	4.1	29
Mono P	0.26	157.1	613.5	7.3	16

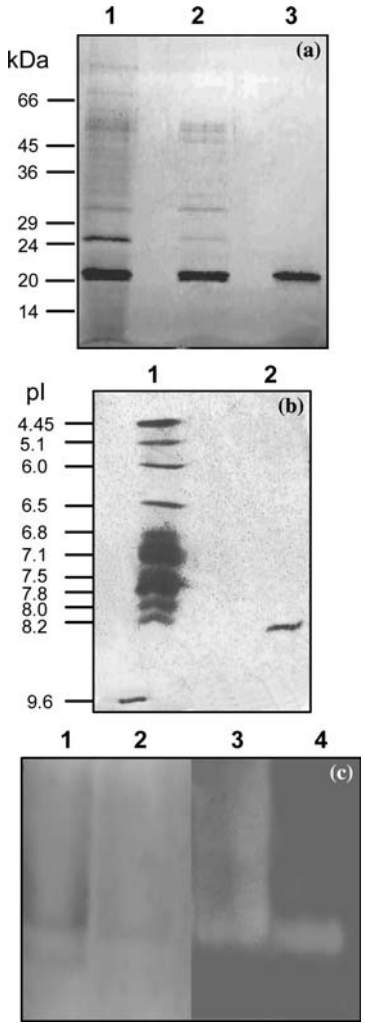

Figure 5. Electrophoresis analysis of purified xylanase. (a) SDS-PAGE of xylanase samples from the three purification steps. Lane 1, freeze-drying concentrated culture filtrate; lane 2, main xylanase fraction from the cation-exchange chromatography step; lane 3, xylanase fraction from the chromatofocusing step. Proteins were stained with Coomassie Brilliant Blue R. (b) Analytical isoelectric focusing (IEF-PAGE) of purified xylanase. Lane 1, IEF markers; lane 2, purified enzyme from the chromatofocusing step. (c) Native PAGE of xylanase samples. Lanes 1, 3, were loaded with freeze-drying concentrated culture filtrate; lanes 2, 4, with purified xylanase from the chromatofocusing step. Lanes 1, 2, stained for protein with Coomassie Brilliant Blue R; lanes 3, 4, zymogram analysis with RBB-xylan-agar gel.

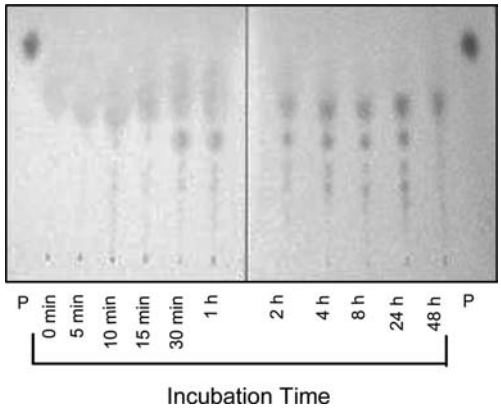

Figure 6. Xylan hydrolysis by purified xylanase. 2% (w/v) xylan reaction mixtures (pH 4.5) were incubated at 37 °C with purified xylanase. At the indicated intervals, samples of the reaction mixture (4 μl) were spotted onto TLC plates, which were developed with ethyl acetate-acetic acid-water (18:7:8, v/v/v) and revealed with $AgNO_3$ reagent. P: xylose. In hydrolysis samples the faster moving spot was identified as xylobiose from its relative mobility with respect to xylose.

significant differences in either time course of enzyme production and isoenzyme composition of the secreted enzyme activities could be found when the races 0 and 5 were compared (Pérez-Artés and Tena 1989, 1990). The results described in the present work indicate that in contrast to their pectinases, Foc races 0 and 5 have a comparable capability for producing extracellular xylanases when grown on xylan or chickpea cell wall as carbon sources. Furthermore, similar differences in time course of released enzyme activity and pH evolution of the culture medium were presented by both races when either carbon sources were compared. Such differences in pH of media and time courses of xylanase production may indicate a possible regulation of Foc xylanase expression by ambient pH as previously established for xylanases from other fungal sources (MacCabe et al. 1998), including *F. oxysporum* f. sp. *lycopersici* (Gómez-Gómez et al. 2002). In relation with this possible pH-dependent regulation of xylanase excretion, it is worth noting that the higher pH value found in 3-day xylan cultures of Foc 0 as compared with those of Foc 5 was accompanied by about three times higher level of excreted xylanase activity (Figure 1).

The xylanase isoenzyme patterns of the two Foc races, as evidenced by preparative IEF analysis, were similar to each other, although minor differences occurred in the relative amounts of the xylanase fractions, presence of additional secondary xylanase peaks and/or in p*I* values of the fractions. Our results show that *F. o. ciceris* is able of secreting both acidic, neutral and alkaline

xylanases and that multiple forms may even be found within each of the above three fractions. Previous reports indicate that very diverse xylanases have been reported for other *formae speciales* or strains of *F. oxysporum* (Alconada and Martínez 1994; Christakopoulos et al. 1996a, b; 1997; Ruiz et al. 1997; Ruiz-Roldán et al. 1999; Cardinale and Matta 2001; Gómez-Gómez et al. 2001, 2002) and, taken with our results, clearly illustrate the high potential of this fungal species to produce an ample array of xylanolytic activities whose expression seems to be highly conditioned by environmental factors (see later).

Referring to the physicochemical characteristics of the major xylanase purified from xylan culture filtrates of both fungal races, the discussion will focus on the following three main properties: molecular weight, p*I* and mode of action. The purified xylanase behaved anomalously on Sephacryl S-200, it being notable that abnormal behaviour of various xylanases on different types of gel filtration media was reported previously in several studies; in these reports it was assumed that ionic or hydrophobic interactions with gel-filtration matrices lead to increased retention and underestimation of MW values (Baker et al. 1977; Dean and Anderson 1991; Royer and Nakas 1991; Holden and Walton 1992; Khasin et al. 1993; Belancic et al. 1995; Lin et al. 1999; Sá-Pereira et al. 2002). Consequently, only the mobility on SDS-PAGE could be used for molecular weight determination. However, the estimated value of MW (21.6 kDa) can be taken with confidence since it is in very good agreement with that found for the catalytic domain of low molecular weight, family 11 xylanases (Törrönen and Rouvinen 1997). Thus, 82 low MW microbial endoxylanases from family 11 of known amino acid sequences presented MW values between 19035 to 26314, with a mean value of 22019 (Sapag et al. 2002). The purified enzyme had an alkaline p*I*, as evidenced by its mobility on analytical IEF-PAGE (indicating a pI near to 8.2) and its lack of fixation at pH 9.0 on the chromatofocusing column, indicating a p$I \geq 9.0$. Both MW and p*I* values were proposed as useful criteria to classify most xylanases into two major groups. Thus, Wong et al. (1988) considered the main groups of acidic and basic xylanases, the first one comprising of high MW (>30 kDa) enzymes with p*I* values between 3.6 and 4.5 and the second one containing low MW (<30 kDa) enzymes with p*I* values ranging from 8.3 to 10. Subsequently, once glycosidases had been classified into several families on the basis of structural homologies (Henrissat and Bairoch 1993), most xylanases were included into two families, i.e., families 10 (formerly F) and 11 (formerly G), which correlated well with the groups of high-MW acidic and low-MW alkaline xylanases, respectively. However, such correspondence is not absolute since family 10 includes some neutral and alkaline p*I* xylanases and family 11 includes some acidic and neutral p*I* xylanases (Törrönen and Rouvinen 1997; Sapag et al. 2002). Thus the MW value, rather than the combination of MW and p*I* values, is the most useful characteristic for the preliminary assignation of a given xylanase into families 10 or 11. Consequently, the major xylanase produced by Foc in xylan cultures could be tentatively characterized as a family 11 alkaline p*I* xylanase. Under similar conditions, *F. o. melonis* produced a xylanase of very high MW (80 kDa) (Alconada and Martínez 1994), whereas *F. o. lycopersici* produced an acidic p*I* xylanase of high MW (40 kDa) (Ruiz et al. 1997) or four alkaline p*I* xylanases of which two were of high MW (34 and 38 kDa, respectively) and two of low MW (19 and 22 kDa, respectively) (Cardinale and Matta 2001). Similarly, the cumin F3 strain of *F. oxysporum* produced one neutral p*I* xylanase of very high MW (60.2 kDa) (Christakopoulos et al. 1996a) and three alkaline p*I* xylanases, one of high MW (38 kDa) and belonging to family 10 (Christakopoulos et al. 1997) and two of low MW (20.8 and 23.5 kDa, respectively) (Christakopoulos et al. 1996b). As noted above, these results suggest that *F. oxysporum* is endowed with a considerable and redundant ability to produce very diverse types of xylanases whose individual expression may be conditioned by different environmental factors. In line with this biochemical evidence, recent molecular genetic studies identified up to four xylanase genes in *F. o. lycopersici*, of which two encode putative family 10 xylanases with predicted p*I*/MW values of 8.9/33 and 6.7/39.3 kDa, respectively, (Ruiz-Roldán et al. 1999) and the other two encode putative family 11 xylanases with predicted p*I*/MW values of 4.3–4.5/25–27 and 6.5/25.6 kDa, respectively (Gómez-Gómez et al. 2001, 2002).

The purified enzyme showed an endohydrolytic mode of action (i.e., more or less random cleavage

at interior sites in the polymer substrate, rather than ordered cleavage at terminal or sub-terminal sites at the non-reducing end of the substrate) as revealed by time course analysis of the products formed in the assay reaction mixture. Endoxylanases producing either xylobiose or a mixture of xylobiose and xylose as main end products from xylan hydrolysis have been described, it being clear from our results that the *F. o. ciceris* enzyme can be included in the former group. It has been suggested that the possession of transglycosidase activity is the possible cause for lack of xylose formation by these enzymes (Royer and Nakas 1991).

In summary, in this present work we established the ability of *F. oxysporum* f. sp. *ciceris* to produce multiple forms of xylanases and compared two pathogenic races of *F. oxysporum* f. sp. *ciceris*, races 0 and 5, with respect to enzyme production. These two races are representative of major differences among this fungal pathogen in symptom type (yellowing, race 0 vs. wilting, race 5), virulence (low virulence, race 0 vs. high virulence, race 5), and evolutionary history (ancient race, race 0 vs. recently evolved race, race 5) (Trapero-Casas and Jiménez-Díaz 1985, Jiménez-Díaz et al. 1994, Navas-Cortés et al. 2000, Jiménez-Gasco et al. 2001). Furthermore, we have purified and partially characterized the main extracellular xylanase produced by both races.

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