

PURIFICATION AND CHARACTERIZATION OF A LOW MOLECULAR WEIGHT XYLANASE FROM SOLID-STATE CULTURES OF *ASPERGILLUS FUMIGATUS* FRESENIUS

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ABSTRACT

A xylan-degrading enzyme (xylanase II) was purified to apparent homogeneity from solid-state cultures of *Aspergillus fumigatus* Fresenius. The molecular weight of xylanase II was found to be 19 and 8.5 kDa, as estimated by SDS-PAGE and gel filtration on FPLC, respectively. The purified enzyme was most active at 55°C and pH 5.5. It was specific to xylan. The apparent K_m and V_{max} values on soluble and insoluble xylans from oat spelt and birchwood showed that xylanase II was most active on soluble birchwood xylan. Studies on hydrolysis products of various xylans and xylooligomers by xylanase II on HPLC showed that the enzyme released a range of products from xylobiose to xylohexaose, with a small amount of xylose from xylooligomers, and presented transferase activity.

Key words: xylan, xylanase, *Aspergillus fumigatus*

INTRODUCTION

Xylan is one of the major constituents of lignocellulosic materials, accounting for approx. 35% of the total dry weight of higher plants (12). The basic molecular structure of xylan is a linear backbone comprised of β -1,4-linked D-xylopyranose residues which, depending on the origin and method of extraction, may be substituted with branches containing mainly acetyl, arabinosyl and glucuronosyl residues (2, 12). The complete cleavage of the complex structure of xylan molecules requires the combined action of β -xylanase (EC 3.2.1.8) and β -xylosidase (EC 3.2.1.37) with debranching enzymes such as α -arabinofuranosidase

(EC 3.2.1.55), acetyl xylan esterase (EC 3.1.1.6) and α -glucuronidase (EC 3.2.1) (2, 22). There is a great interest in the enzymatic hydrolysis of xylan because of possible applications in ruminal digestion, waste treatment, fuel and chemical production, and paper manufacture (2, 12, 27). This work reports the purification and some properties of xylanase II from solid-state cultures of the fungus *Aspergillus fumigatus* Fresenius.

MATERIALS AND METHODS

Organism and Enzyme Production. *A. fumigatus* Fresenius was isolated from a hot fountain in Brasil (Caldas Novas, Goiás) and identified by

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specialists of the Mycology Department from the Universidade Federal de Pernambuco (Brazil). For production of xylanase activity, *A. fumigatus* Fresenius was cultured at 42°C for 7 days in a solid-state medium containing wheat bran (27). After the growth procedure, the contents of the flasks were extracted with 750 ml of 25 mM sodium acetate buffer, pH 5.0 and placed under shaking (120 rpm) at room temperature for 3 h. The resulting crude extract was centrifuged for 30 min at 10,400 g and 5°C, filtered and stored at 5°C for subsequent use as source of xylanase activity.

Enzyme Assays. Xylanase activity was determined by mixing 25 µl of enzyme solution with 50 µl of oat spelt xylan (3.0-50 mg/ml) in 100 mM sodium acetate buffer, pH 5.0 at 50°C for 30 min. The release of reducing sugar was measured using the dinitrosalicylic reagent method (19, 24). Xylanase activity was expressed as µmol reducing sugar formed min⁻¹ ml⁻¹ enzyme solution, i.e., as IU ml⁻¹ and IU mg⁻¹ protein. β-Glucanase, carboxymethyl-cellulase and β-mannanase assays were performed in the conditions as described above. The activity against filter paper was measured as described by Mandels *et al.* (18). β-Xylosidase, α-arabinofuranosidase, β-glucosidase and β-mannosidase activities were determined as reported elsewhere (24). For the kinetic experiments, soluble and insoluble samples from oat spelt and birchwood xylans were used as substrates in a concentration range of 0.05-8.0 mg/ml. The substrates were prepared as described by Filho *et al.* (11, 13). K_m and V_{max} Values were estimated from Michaelis-Menten equation with a non-linear regression data analysis program (17). The determination of optimum temperature of xylanase II was carried out in the temperature range of 30 to 90°C in 100 mM sodium acetate buffer, pH 5.0. To determine the optimum pH of xylanase II activity at 50°C, the range was from 3.0 to 8.0. McIlvaine type buffer systems were adjusted to the same ionic strength with KCl (7). The temperature stability of xylanase II was determined by pre-incubating the enzyme at 55°C. At various time periods, aliquots were withdrawn and the residual activity was measured under standard conditions.

Protein Concentration. Protein concentration was measured by the method of Petterson (20), using bovine serum albumin as standard.

Enzyme Purification. All purification steps were carried out at 4°C unless otherwise specified. The

crude extract was concentrated by ultrafiltration using an Amicon system with a 10 kDa cut-off point membrane (PM 10). Aliquots of the ultrafiltrate were fractionated by gel filtration on Sephadex G-50 (2.3 x 42 cm), pre-equilibrated with 100 mM sodium acetate buffer, pH 5.0. Fractions of 4.0 ml were collected at a flow rate of 12 ml/h. Fractions with xylanase activity were pooled, dialyzed against 10 mM sodium phosphate buffer, pH 7.2, and loaded onto a Econo-Pac CHT-II column (1.3 x 4.1 cm), equilibrated with the same buffer. Fractions of 2.0 ml were collected at a flow rate of 30 ml/h by washing the column with buffer followed by a linear gradient of sodium phosphate buffer (10-400 mM), pH 7.2, and those with xylanase activity were pooled and dialyzed against 20 mM sodium phosphate buffer, pH 7.0. The dialyzed solution was applied to a Econo-Pac High Q column (1.3 x 4.1 cm) pre-equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The column was washed with the same buffer and eluted with a linear gradient of NaCl (0-1 M). Fractions of 2.0 ml were collected at a flow rate of 120 ml/h. Fractions containing xylanase activity were pooled and dialyzed against 20 mM sodium phosphate buffer, pH 6.5. Finally, further enzyme purification was performed in the same column equilibrated with 20 mM sodium phosphate buffer, pH 6.5 at a flow rate of 120 ml/h. The residual protein was eluted with a NaCl linear gradient from 0 to 1 M. Fractions corresponding to xylanase activity were pooled, concentrated by freeze-drying and stored for later use at 4°C.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (16) using 12% gels. After electrophoresis, protein bands were silver stained by the method of Blum *et al.* (3). The molecular weight of xylanase II was estimated by SDS-PAGE and gel filtration on a fast protein performance liquid chromatography system (FPLC) using MW marker kits from Sigma Chemical Co., USA. The FPLC column (Superose 12) was equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The elution was performed at a flow rate of 30 ml/h.

Hydrolysis Products. The reaction mixture containing 100 µl of enzyme solution (20 µg/ml) and 500 µl of 0.2% xylan in distilled water was incubated for 16 h at 28°C with shaking at 100 rev./min. The reaction mixture from above was stopped by heating in boiling water and centrifuged at 3,000 x g for 5

min. The determination of hydrolysis products was made by high performance anion exchange chromatography coupled with pulsed amperometric detection (Dionex Corp., USA), as described previously (11, 22, 23). The analysis of the hydrolysis products of xylooligomers by xylanase IIa was also performed as described above. However, the hydrolysis of xylooligomers was determined following 1 to 2 h of incubation at 40°C.

Chemicals. Oat spelt xylan and birchwood xylan, were from Sigma Chemical Co., USA. All other chemicals were analytical grade reagents. Deacetylated and acetylated xylylans were obtained by dimethylsulfoxide extraction (DMSO) of beechwood and wheat straw holocelluloses, respectively (23). Xylan extracted by HCl from the seaweed *Palmaria palmata* was a gift from Maria G. Tuohy (University College Galway, Ireland). Xylooligosaccharides were prepared as described before (21).

RESULTS AND DISCUSSION

A xylanase was isolated from the xylan-degrading enzyme system of *A. fumigatus* Fresenius, and purified to apparent homogeneity by a combination of ultrafiltration and chromatographic procedures. The purification steps of xylanase II are summarized in Table 1. The ultrafiltration experiment showed that a xylanase activity was found to be present in the ultrafiltrate. For further purification, the ultrafiltrate was subjected to gel filtration chromatography on Sephadex G-50 (Fig. 1). The sample elution resulted in the separation of two peaks

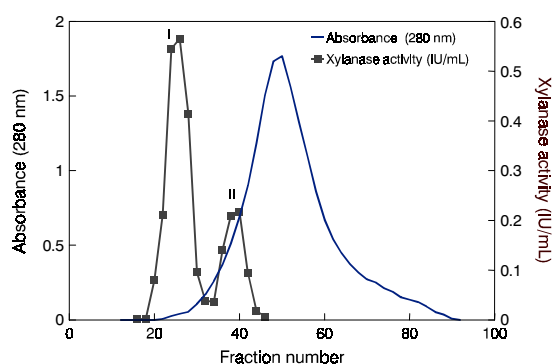


Figure 1. Fractionation on Sephadex G-50 of *A. fumigatus* Fresenius ultrafiltrate. For experimental details see text.

of xylanase activity (I and II). The second peak, designated xylanase II, was used for further purification by hydroxylapatite and anion-exchange chromatographies (results not shown). In both purification procedures, xylanase II was eluted in the pre-gradient wash fractions. The purification step procedures provided an apparently homogeneous preparation of xylanase II, as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme migrated as a single 19 kDa band on SDS-PAGE stained with silver nitrate (Fig. 2). However, its apparent size by gel filtration was 8.5 kDa, indicating a physical interaction between the enzyme and chromatography resin (result not shown). This is in agreement with the results obtained for xylan-degrading enzymes from *T. harzianum*, *T. reesei*, *A. oryzae*, *A. fumigatus* VTT-D-71002 and *Bacillus* sp (1, 8, 26). A small amount of carbohydrate was found when the purified

Table 1. Summary of the purification of β -xylanase II from *A. fumigatus* Fresenius.

Step	Total protein (mg)	Total activity (IU)	Specific activity (IU mg ⁻¹)	Yield (%)	Purification factor
Crude Extract	196.78	1,234.0	6.27	100	1.0
Ultrafiltration	37.41	210.21	5.62	17.05	0.90
Sephadex G-50	19.23	90.0	4.67	7.30	0.74
Econo-Pac CHT-II	6.13	12.0	2.0	1.0	0.31
Econo-Pac High Q pH 7.0	2.82	6.04	2.14	0.50	0.34
Econo-Pac High Q pH 6.5	0.45	3.21	7.10	0.26	1.32

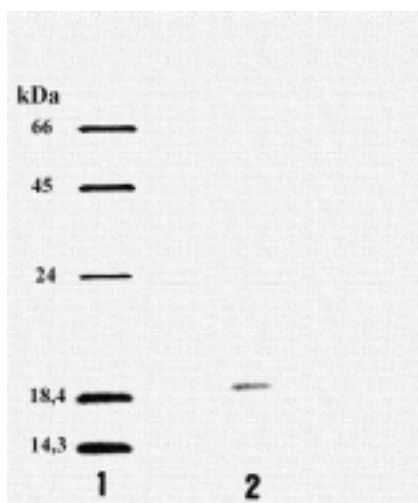


Figure 2. SDS-PAGE (12%) of β -xylanase II from *A. fumigatus* Fresenius stained with silver nitrate. Lane 1, molecular weight standards (from the top): bovine serum albumin (66 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa), β -lactoglobulin (18.4 kDa), lysozyme (14.3 kDa); lane 2, xylanase II.

xylanase II from *A. fumigatus* Fresenius was assayed with the phenol-sulfuric acid method (9). The low yield and fold-purification values obtained for xylanase II from *A. fumigatus* were probably underestimated. This phenomenon is often described during purification of xylan-degrading enzymes produced by fungi (10, 13, 14). At least one major and some minor peaks of xylanase activities were also detected in the ultrafiltrate during the purification procedure. Besides, the ultrafiltration procedure retained most of the bulk of xylan-degrading activities in the retentate. Fractionation of the retentate by gel filtration, adsorption and ion-exchange chromatography techniques showed the presence of, at least, nine different xylanase activities. These enzymes may act synergistically with xylanase II to effect the complete hydrolysis of xylan (10).

Xylanase II was optimally active at pH 5.5 and 55°C. This optimum pH value was also described for xylanases from *A. sydowii* MG49 and *A. niger* (15, 25). The purified enzyme showed a half-life of 80 min. when incubated at 55°C and pH 7.0 (Fig. 3). The purified enzyme was not active on CMC, filter paper, para-nitrophenyl β -D-xylopyranoside, para-nitrophenyl α -L-arabinofuranoside, para-nitrophenyl β -D-mannopyranoside, para-nitrophenyl β -D-glucopyranoside, β -laminarin and β -mannan. In addition, the enzyme was specific for xylan. The K_m values for soluble xylan from oat spelt were much

higher than the insoluble one (Table 2). This might suggest a steric hindrance due to the presence of side-chains groups in soluble xylan. Conversely, the hydrolysis of soluble birchwood xylan by xylanase II was more effective than when the enzyme was incubated with the insoluble xylan, suggesting that the presence of a particular type of substituent (acetyl group) in the vicinity would be a requirement for the action of xylanase II (4). The hypothesis of the substituents are probably situated in regions of the polysaccharide distant from the unsubstituted portions can not be discarded. Debranching activity was also found in some xylanases (2). The K_m values for xylanase II were lower than the values found for xylanases from *F. oxysporum* and *P. capsulatum* (5, 6, 11).

Table 2. Some kinetic properties of the purified β -xylanase II produced by *A. fumigatus* Fresenius.

Substrate	K_m (mg/ml)	V_{max} (IU/ml)
Soluble oat spelt xylan	5.72	2.34
Insoluble oat spelt xylan	3.01	1.41
Soluble birchwood xylan	2.19	1.55
Insoluble birchwood xylan	5.19	1.89

The predominant hydrolysis products of birchwood and deacetylated xylans by xylanase II ranged from xylobiose to xylohexaose (Table 3). Xylanase IIa was not able to release xylose from xylan molecules, suggesting an endomechanism. Xylan from seaweed (*Palmaria palmata*), a β -1,3;1,4-linked polymer, was degraded to a mixture of xylobiose and xylotriose, while xylobiose to xylopentaose were released from oat spelt xylan. The high proportion of acetyl groups linked to xylose residues in acetylated xylan was probably a steric obstacle to xylanase II activity (12). The purified xylanase did not show any activity against xylotriose and xylo-tetraose. The hydrolytic capacity of β -xylanase increased with increasing chain length of xylooligomers (Table 3). Xylose, xylobiose and xylo-tetraose were removed from xylopentaose. The enzyme showed transferase activity when incubated with xylohexaose. The purified enzyme was able to produce higher molecular weight transfer products. Transferase activity has been reported for some other xylan-degrading enzymes from *Aspergillus* (1, 5).

In conclusion, the fungus *A. fumigatus* Fresenius produces multiple forms of xylanases. The purified xylanase II is a 19 kDa enzyme with an acidic

Table 3. Hydrolysis products of xylans and xylooligomers by xylanase II from *A. fumigatus* Fresenius. Abbreviations: X1, xylose; X2, xylobiose; X3, xylotriose; X4, xylo-tetraose; X5, xylopentaose; X6, xylohexaose; ND, not detected.

Substrate	Hydrolysis Products
Oat spelt xylan	X2, X3, X4, X5
Birchwood xylan	X2, X3, X4, X5, X6
Acetylated xylan	ND
Deacetylated xylan	X2, X3, X4, X5, X6
Seaweed xylan	X2, X3
Xilotriose ^a	ND
Xilotriose ^b	ND
Xylo-tetraose ^a	ND
Xylo-tetraose ^b	ND
Xylopentaose ^a	X1, X2, X4
Xylopentaose ^b	X1, X2, X4
Xylohexaose ^a	X3, X4, X5
Xylohexaose ^b	X1, X3, X4, X5

^aOne hour incubation; ^btwo hours incubation

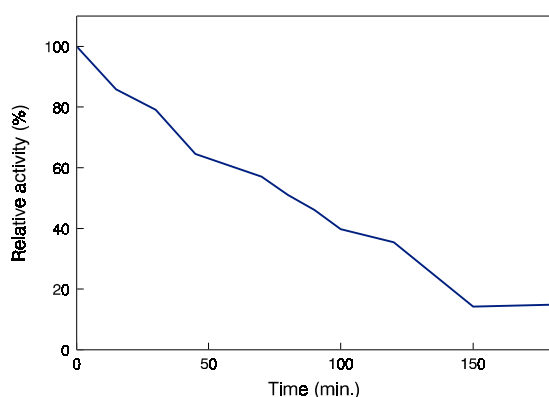


Figure 3. Stability of purified xylanase II from *A. fumigatus* Fresenius at 55°C.

optimum pH and thermostability. It seems to belong to the group of specific xylanases with an endo-acting mechanism.

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RESUMO

Purificação e caracterização de uma xilanase de baixo peso molecular de culturas de estado sólido de *Aspergillus fumigatus* Fresenius

Uma enzima xilanolítica (xilanase II) foi purificada a partir de culturas de estado sólido de *Aspergillus fumigatus* Fresenius. O peso molecular de xilanase II foi estimado em 19 e 8,5 kDa por SDS-PAGE e FPLC, respectivamente. A enzima purificada apresentou maior atividade a 55°C e pH 5,5, além de hidrolisar especificamente xilana. Os valores aparentes de K_m e V_{max} de xilanas solúveis e insolúveis, isoladas de cereal e madeira, mostrou que xilanase IIa foi mais ativa em xilana solúvel de madeira. Estudos sobre produtos de hidrólise de xilanas e xilooligômeros por xilanase II em HPLC revelou que a enzima liberou uma variedade de xilooligômeros (xilobiose-xilohexose) e uma pequena quantidade de xilose a partir de xilooligômeros, apresentando atividade de transferase.

Palavras-chave: xilana, xilanase de baixo peso molecular, purificação da enzima, caracterização da enzima.

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