

Purification and biochemical characterization of an endoxylanase from *Aspergillus versicolor*

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Abstract

An endoxylanase (β -1,4-xylan xylanohydrolase, EC 3.2.1.8) was purified from the culture filtrate of a strain of *Aspergillus versicolor* grown on oat wheat. The enzyme was purified to homogeneity by chromatography on DEAE-cellulose and Sephadex G-75. The purified enzyme was a monomer of molecular mass estimated to be 19 kDa by SDS-PAGE and gel filtration. The enzyme was glycoprotein with 71% carbohydrate content and exhibited a *pI* of 5.4. The purified xylanase was specific for xylan hydrolysis. The enzyme had a K_m of 6.5 mg ml⁻¹ and a V_{max} of 1440 U (mg protein)⁻¹. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Xylan is, after cellulose, the most abundant carbon source present in wood and agricultural residues. Also, it is the major hemicellulosic polysaccharide present in plant cell walls. This complex heteropolysaccharide consists of a main chain of β -1,4-D-xylanpyranosyl residues and branches of neutral or uronic monosaccharides and oligosaccharides [1]. In nature xylan is completely hydrolyzed to monosaccharides by the combined action of a family

of extracellular isoenzymes produced by fungi or bacteria [2,3]. Recently, the interest in xylanolytic enzymes has increased, due to their use in several industrial processes, such as bleaching of kraft pulp, bio-conversion of lignocellulose-derived sugars into fuel, processing food including bread-making and clarification of beer and juice [4]. The prerequisite for the application of xylanase in the paper industry is that the enzyme preparation must be free of cellulase activity [1,5]. We isolated from Brazilian soil a strain of *Aspergillus versicolor*, which produced high xylanase and low cellulase levels [6]. In this report we describe the purification procedure, and some biochemical properties of an extracellular xylanase obtained from this strain of *Aspergillus*.

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2. Materials and methods

2.1. Culture conditions

The *A. versicolor* strain was grown in liquid Vogel's [7] medium, containing 1.0% (w/v) wheat bran as the sole carbon source. Erlenmeyer flasks containing 25 ml of medium were incubated for 120 h at 30°C in a reciprocating shaker.

2.2. Enzyme assay

Xylanase activity was assayed at 55°C using 0.6% (w/v) oat spelt xylan as substrate, in 50 mM sodium phosphate buffer (pH 6.0). Reducing sugars release was determined using the dinitrosalicylic acid method [8]. An enzyme unit is the amount of enzyme that produces 1 μmol of xylose equivalent per minute.

2.3. Purification of extracellular xylanase

Culture filtrates (280 ml) were collected by filtration on paper (Whatman No. 1) and dialyzed overnight against 50 mM Tris-HCl (pH 7.0). The dialyzed sample was applied to a DEAE-Sephadex A-50 column (20.0 × 1.8 cm) equilibrated with the dialysis buffer. Xylanase activity was resolved into two peaks, named form I and form II (Fig. 1). Form I did not bind to the resin and was eluted in the flow-through of the column (flow rate 90 ml per min). The column was then washed with 150 ml of equilibrating buffer. Form II was eluted with a gradient of 0–0.7 M sodium chloride in 50 mM Tris-HCl (pH 7.0). Form I was pooled, dialyzed against water, lyophilized, redissolved in 50 mM sodium phosphate (pH 7.0) and applied to a Sephadex G-75 column (70.0 × 1.8 cm) equilibrated with the same buffer. Fractions containing xylanase activity were pooled, dialyzed against distilled water and lyophilized.

2.4. Analytical methods

Total neutral carbohydrate was estimated by the method of Dubois [9]. Protein content was estimated by the method of Lowry [10] using bovine serum albumin as standard. Hydrolysis products from xylan were analyzed by thin-layer chromatography on silica gel G-60, using ethylacetate/acetic acid/formic acid/water (9:3:1:4, by volume) as the mobile phase system. Sugars were detected with 0.2% (w/v) orcinol in sulfuric acid/methanol (10:90).

3. Results and discussion

3.1. Purification of xylanase form I

Form I corresponded to about 55% of total xylanase activity (Table 1). After the Sephadex G-75 step, the specific activity of xylanase was about 635 ± 95 U (mg protein)⁻¹ ($n=6$) and a 19-fold purification was achieved (Table 1). The purified xylanase migrated slowly in non-denaturing 7.5% PAGE at pH 8.9 but showed a single protein band when run in 10% SDS-PAGE (Fig. 2).

3.2. Molecular properties

Sephadex G-100 gel filtration and SDS-PAGE showed that xylanase form I had a molecular mass of about 19 kDa (Fig. 2), suggesting that the enzyme was a monomeric protein. Electrofocusing of the purified xylanase form I revealed a *pI* of about 5.4, which should be an exception since Wong et al. [12] suggested that the acidic endoxylanases could be grouped into those of MW above 30 kDa. The carbohydrate content of the purified enzyme form I was estimated to be 71%, which can explain the slow migration in non-denaturing 7.5% PAGE at pH 8.9. The presence of carbohydrate is reported for other

Table 1
Purification of a xylanase from *A. versicolor*

Step	Total protein (mg)	Total units	Specific activity (U mg ⁻¹)	Yield (%)	Purification (-fold)
Dialyzed	189	6386	33.79	100	1.0
DEAE-Sephadex (peak I)	20	3490	174.50	55	5.2
Sephadex G-75	2	1270	635.0	19.9	18.8

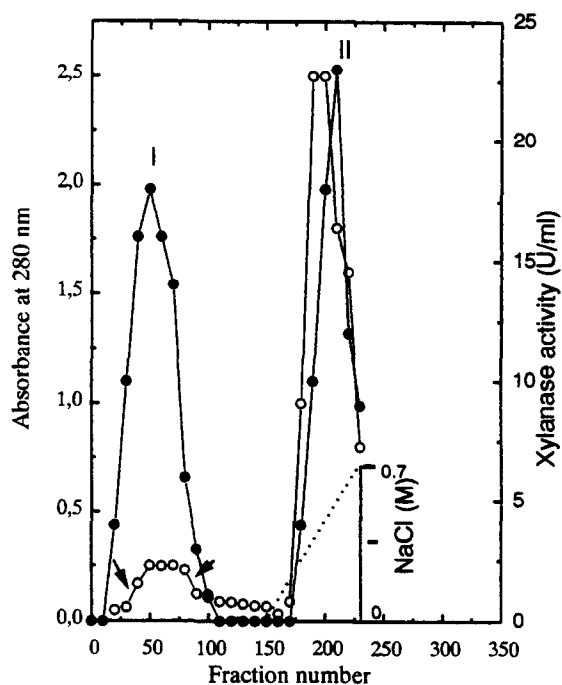


Fig. 1. DEAE-cellulose chromatography of dialyzed culture filtrate from *A. versicolor*. The arrows indicate the pool of fractions used for purification of xylanase form I. ●, xylanase activity; ○, $A_{280\text{nm}}$; broken line, sodium chloride gradient.

microbial xylanases [2] and particularly in *Humicola grisea* var. *thermoidea* which exhibits a high carbohydrate content [12].

3.3. Effect of temperature and pH on activity and stability

The temperature for maximum activity was estimated to be 55°C, and 85% and 90% of maximal activity were found at 50°C and 60°C, respectively. The temperature optimum of xylanase form I of *A. versicolor* was comparable with that of xylanases from many mesophilic and thermophilic fungi, ranging from 40 to 60°C [11,12]. The purified xylanase showed good stability up to 60 min at 50°C, when incubated in aqueous solution. At 60°C its half-life was about 17 min. However, in the presence of substrate the half-life increased about 2.2-fold. The pH for maximum activity was estimated to be 6.0, and 91% and 89% of maximal activity were found at 5.5 and 6.5, respectively. The pH optimum for the *A.*

versicolor xylanase was close to that of other microbial xylanases [11]. The purified enzyme was stable up to 60 min in pH range of 4.0–8.0.

3.4. Effect of metal ions and several compounds on xylanase activity

Zn^{2+} , Co^{2+} , Mn^{2+} , Ca^{2+} , NH_4^+ and Ba^{2+} up to 10 mM concentration had no significant effect on xylanase activity. Pb^{2+} and Hg^{2+} at 2.0 mM concentration inhibited the enzyme activity 28% and 89%, respectively. At 10 mM Pb^{2+} and Hg^{2+} inhibited the enzyme 58% and 100%, respectively. Fe^{2+} inhibited 16% and 48%, at 2.0 mM and 10 mM, respectively. Cu^{2+} and Al^{2+} at 10 mM inhibited 91% and 44%, while at 2 mM the enzyme activity was not significantly affected by these metals. SDS and EDTA at 10 mM inhibited the enzyme 69% and 51%, respectively. Cysteine and dithiothreitol at 1 mM had no effect on the xylanase activity.

3.5. Kinetic studies

K_m and V_{max} values were estimated using oat-spelt

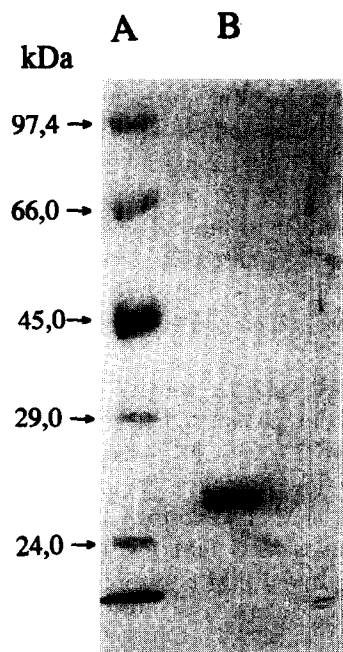


Fig. 2. SDS-PAGE (10%) of purified xylanase. A: Sigma molecular mass markers; B: 20 μg xylanase was applied. The gel was stained with Coomassie brilliant blue.



Fig. 3. Thin-layer chromatography of the products of xylan hydrolysis by purified xylanase. Lanes 1, 2, 3 and 4 are from 10-, 30-, 60- and 1440-min samples, respectively. Lanes 5 and 6 represent xylobiose and xylan standards, respectively.

xylan as substrate. The purified xylanase exhibited typical Michaelis-Menten kinetics with K_{m} and V_{max} values of $6.5 \pm 1.2 \text{ mg ml}^{-1}$ and $1440 \pm 245 \text{ U (mg protein)}^{-1}$ ($n=6$), respectively. These values were quite similar to those described for *Aspergillus nidulans* acidic xylanase [13].

3.6. Substrate specificity

The purified xylanase activity was rather specific, hydrolyzing only xylan. It had no activity when incubated up to 2 h with 1% avicel, 0.5% carboxymethylcellulose (low viscosity), 1% soluble starch, 5 mM cellobiose, 1 mM xylobiose, 5 mM *p*-nitrophenyl- β -D-xylopyranoside, 5 mM *p*-nitrophenyl- β -D-galactopyranoside and 5 mM *p*-nitrophenyl- β -D-glucopyranoside.

3.7. Hydrolysis pattern

The products of a time-course hydrolysis of xylan

by the purified xylanase were analyzed by thin-layer chromatography (Fig. 3). At initial and long periods of incubation the reaction products contained only xylo-oligosaccharides without detectable xylobiose or xylose. This pattern of hydrolysis classified the *A. versicolor* xylanase form I as an endoenzyme (β -1,4-xylan xylanohydrolase, EC 3.2.1.8).

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