Penicillium purpurogenum produces several xylanases: Purification and properties of two of the enzymes

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Abstract

The fungus *Penicillium purpurogenum* produces several extracellular xylanases. The two major forms (xylanases A and B) have been purified and characterized. After ammonium sulfate precipitation and chromatography in Bio-Gel P 100, xylanase A was further purified by means of DEAE-cellulose, hydroxylapatite and CM-Sephadex, and xylanase B by DEAE-cellulose and CM-Sephadex. Both xylanases showed apparent homogeneity in SDS-polyacrylamide gel electrophoresis. Xylanase A (33 kDa) has an isoelectric point of 8.6, while xylanase B (23 kDa) is isoelectric at pH 5.9. Antisera against both enzymes do not cross-react. The amino terminal sequences of xylanases A and B show no homology. The results obtained suggest that the enzymes are produced by separate genes and they may perform different functions in xylan degradation.

Keywords: P. purpurogenum; Xylanase; Enzyme purification; Amino acid sequence, similarity

1. Introduction

Xylan is the principal component of hemicelluloses from annual plants and hardwoods. It consists of a linear chain of xylose residues joined by β $(1 \rightarrow 4)$ glycosidic linkages with various types and number of substitutions depending on its origin. Known substituents are O-acetyl groups, L-arabinose, D-O-methyl glucuronic acid and phenolic compounds such as coumaric and ferulic acids (Joseleau et al., 1992).

Xylan biodegradation is performed by enzymes produced by bacteria and fungi known collectively as xylanases. Due to the complexity of xylan structure, its hydrolysis is accomplished by the concerted action of several enzymes differing in their bond-breaking specificity (Biely, 1985).

Xylanases are finding increasing applications in biotechnology, particularly in cellulose pulp biobleaching (Viikari et al., 1994). Although considerable work has been carried out on the enzymology of xylan degradation, the role and specificity of the different components of the xylanase system remains unclear (Puls and Poutanen, 1989; Maringer et al., 1995). A better understanding of

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the hydrolytc properties of these enzymes would be valuable to stimulate their industrial use.

Penicillium purpurogenum is an active cellulase and xylanase producer (Steiner et al., 1994). In this work, the purification and properties of two endoxylanases obtained from culture filtrates of the fungus is reported. Important differences are observed in the properties of both enzymes, suggesting that they are the product of separate genes and that they may perform different roles in xylan degradation. Part of this work has been presented elsewhere (Eyzaguirre et al., 1992).

2. Materials and methods

2.1. Enzyme source

P. purpurogenum was grown as described previously (Hidalgo et al., 1992), using wheat straw (1%) as carbon source and a mixture of urea (0.3 g l^{-1}), ammonium sulfate (1.4 g l^{-1}) and neopeptone (0.75 g l^{-1}) as nitrogen source. After 8 d of growth, the cultures were centrifuged (15 min at 27000 × *g*) and the supernatant was concentrated by ultrafiltration using a Pellicon PTGC membrane (Millipore Corp.) with cutoff at 10000.

2.2. Purification of xylanases A and B

The concentrated supernatant was fractionated with ammonium sulfate (20–70%). The precipitate was resuspended in 20 mM acetate buffer pH 5.5 and subjected to gel filtration in a 1.7 × 25 cm column of Bio-Gel P-100 (Bio-Rad) using the same buffer. Two major xylanase peaks were obtained which were named A and B.

Xylanase A was further purified using a 1.7 × 24.5 cm DEAE-cellulose DE-32 column (Whatman) equilibrated with 20 mM sodium phosphate buffer pH 7.0. The enzyme eluted in the column washings. The active fractions were pooled, and after adjusting pH to 5.5 with 0.5 M acetic acid, they were loaded onto a 1 × 24 cm hydroxylapatite (Bio-Gel HPT; Bio-Rad) column previously equilibrated with 20 mM sodium acetate buffer pH 5.5. The column was washed with the same buffer and the activity was recovered in the washings. The pH of the pooled active fractions was adjusted to 4.7 with 0.5 M acetic acid. The enzyme was then subjected to chromatography on a 1 × 40 cm CM-Sephadex C-25 column (Pharmacia) equilibrated with 20 mM sodium acetate buffer pH 4.7. The column was washed with 2 vol. of the same buffer followed by 2 vol. of the buffer containing 0.1 M NaCl. Elution was achieved using a linear gradient (100 ml) of 0.1 to 0.5 M NaCl in the buffer.

The purification of xylanase B was continued by means of ion exchange chromatography in a DEAE-cellulose (Cellex D, Bio-Rad) column (1.7 × 26 cm) equilibrated with 20 mM sodium phosphate buffer pH 6.5. The column was washed with buffer and the activity was recovered in the washings. The active fractions were pooled and adjusted to pH 4.0 by means of 0.5 M acetic acid and placed in a 1.7 × 25 cm CM-Sephadex C-50 (Pharmacia) column equilibrated in 20 mM acetate buffer pH 4.0. After washing the column with 2 vol. of the buffer, the enzyme was eluted using a salt gradient (NaCl from 0.1 to 0.5 M, 160 ml) in the buffer.

2.3. Enzyme assays

Endo-β-1,4-xylanase (EC 3.2.1.8) was routinely assayed at 50° C and pH 5.3 for 5 min following the procedure of Bailey et al. (1992) with 1% birchwood xylan (Sigma) as substrate. Reducing power was detected with dinitrosalicylate, using xylose as standard. When long-term xylan hydrolysis was studied, incubations were done for 24 h. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of xylose per min under the above conditions. Mannanase (EC 3.2.1.78) and endoglucanase (EC 3.2.1.4) activities were assayed as described for xylanase using 1% locust bean mannan or carboxymethyl cellulose as substrates. Acetyl esterase was measured according to Poutanen and Sundberg (1988) using α-naphtyl acetate; arabinofuranosidase (EC 3.2.1.55) using *p*-nitrophenyl-α-L-arabinofuranoside (Tagawa and Kaji, 1988) and β-xylosidase (EC 3.2.1.37) by means of *p*-nitrophenyl-β-D-xylopyranoside (Poutanen et al., 1987). All these substrates were

supplied by Sigma. Zymograms were performed according to Lee et al. (1993).

2.4. Preparation of hemicelluloses

Arabinoxylan (hemicellulose A) and arabinoglucuronoxylan (hemicellulose B) were prepared from wheat straw following the method of Adams (1965).

2.5. Protein determination

The presence of protein was monitored in the column eluates measuring absorbance at 280 nm. Protein content was determined by a modification of the Lowry procedure as described by Tan et al. (1984).

2.6. Polyacrylamide gel electrophoresis

Electrophoresis under denaturing conditions (SDS-PAGE) was performed by the method of Laemmli (1970) using 12% acrylamide. Gels were stained for proteins using Coomassie blue (Bollag and Edelstein, 1991) silver nitrate (Wray et al., 1981) or a combination of both. The following molecular weight standards (Sigma) were used: bovine serum albumin, 66 000; ovalbumin, 43 000; glucose-6-phosphate dehydrogenase, 36 000; carbonic anhydrase, 29 000; trypsinogen, 24 000 and trypsin inhibitor, 20 000.

2.7. Isoelectrofocusing

This technique was performed using SERVALYT Precotes 3–10 (Serva) following the manufacturers' instructions. After running the gels, enzyme activity was detected on an overlay of agarose containing Remazol brilliant blue linked to xylan (RBB-xylan) (Biely et al., 1985). Isoelectric points were established by comparison with appropriate standards, which were identified by staining the gel with Coomassie blue.

2.8. Immunological methods

Antisera against purified xylanases A and B were prepared as follows. Young rabbits were injected subcutaneously either 250 μg xylanase B or 500 μg xylanase A in Freund's complete coadjuvant (Difco). After 30 days (xylanase A) or 45 days (xylanase B), similar doses of enzyme were injected in Freund's incomplete coadjuvant. Ten days later the rabbits were bleeded to obtain the sera.

Immunoblots were performed according to Towbin et al. (1979). Alkaline phosphatase linked to anti-IgG antibody (Sigma) was used to detect the antigen–antibody complexes.

2.9. Amino acid sequence

Samples for sequencing were prepared according to the method of Matsudaira (1987). N-terminal sequencing of xylanases A and B was performed by standard automated gas-phase Edman degradation.

3. Results

3.1. Presence of multiple xylanase forms

A zymogram of culture supernatant of *P. purpurogenum* (Fig. 1, lane 3) shows several activity

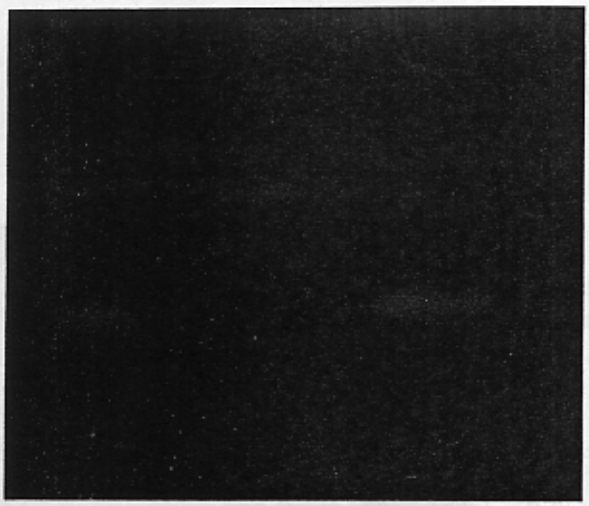
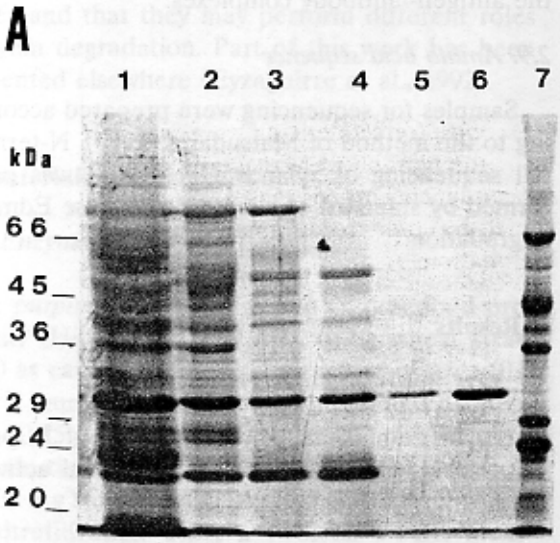
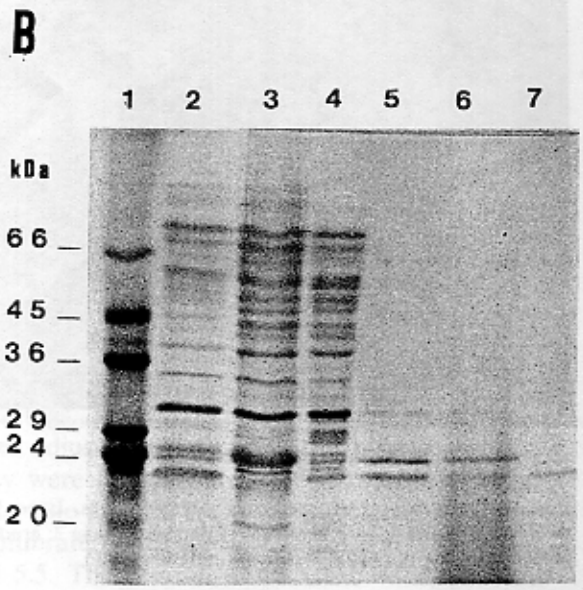


Fig. 1. Zymogram of *P. purpurogenum* xylanases. Lane 1: purified xylanase B; lane 2: purified xylanase A; lane 3: crude culture supernatant (wheat straw as carbon source).

bands, indicating the presence of multiple forms of xylanases. The forms of lower molecular weight, called xylanases A and B (lanes 2 and 1 of Fig. 1) have been purified (see below). The other forms have not been further characterized.





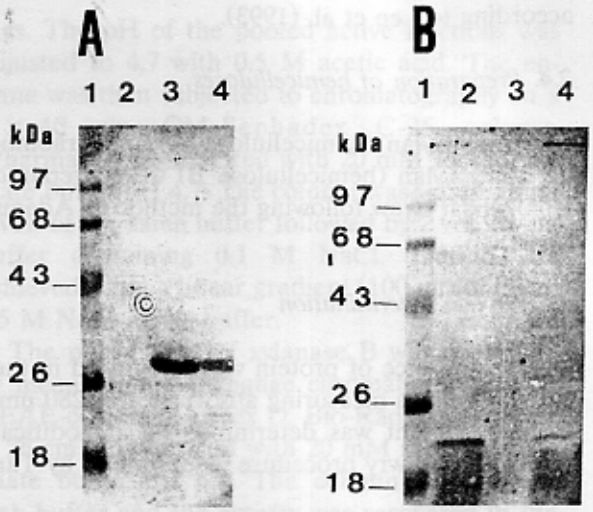


Fig. 3. Western blot of xylanases A and B. Panel A: antiserum against xylanase A; panel B: antiserum against xylanase B. Lane 1: pre-stained molecular mass standards; lane 2: xylanase B; lane 3: xylanase A; lane 4: culture supernatant.

3.2. Purification of xylanases A and B

The two forms of xylanase were separated from each other by gel filtration and were further purified as described in Materials and methods. Tables 1 and 2 summarize their purification. The purity of the isolated enzymes was established by SDS-PAGE (Fig. 2). Both enzymes were free of endoglucanase, mannanase, acetyl esterase, β-xylosidase and arabino-furanosidase activities.

Fig. 2. SDS-polyacrylamide gel electrophoresis of the various steps in the purification of the xylanases. Panel A, xylanase A. Lane 1: concentrated culture supernatant; lane 2: fraction A from the Bio-Gel P-100 column; lane 3: DEAE-cellulose column eluate; lane 4: hydroxylapatite column eluate; lanes 5 and 6: Sephadex CM-C25 column eluate; lane 7: molecular mass standards. The gel was stained with silver nitrate and Coomassie brilliant blue. Panel B, xylanase B. Lane 1: molecular mass standards; lane 2: concentrated culture supernatant; lane 3: ammonium sulfate precipitation; lane 4: fraction A of the P-100 column; lane 5: fraction B of the Bio-Gel P-100 column; Lane 6: DEAE-cellulose column eluate; lane 7: CM Sephadex C-50 eluate. The gel was stained with silver nitrate and Coomassie brilliant blue.

Table 1
Summary of the purification of xylanase A

Purification step	Volume (ml)	Proteins (mg ml^{-1})	Total activity (units)	Spec. activity (U mg^{-1} prot.)
Crude supernatant	312	0.51	9778	61.2
$(NH_4)_2SO_4$	4.6	16.2	5998	80.5
Bio-Gel P-100 (fraction A)	14.2	1.67	2823	119
DEAE-cellulose	20.6	0.36	1581	213
Hydroxylapatite	51.7	0.03	972	606
CM-Sephadex	96.6	0.006	561	966

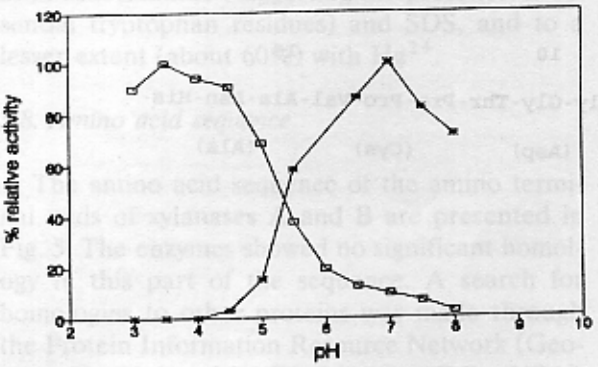


Fig. 4. pH-activity curves for the purified xylanases A and B. The following buffers were used: pH range 3 to 5.5, 50 mM acetate; pH range 6 to 8, 50 mM phosphate. Xylanase A: *; xylanase B: □. The activity of the enzymes at pH 5.3 was: xylanase A, 966 U mg^{-1} prot. and xylanase B, 199 U mg^{-1} prot.

3.3. Molecular properties

The molecular mass of xylanases A and B deduced from SDS gels was found to be 33 kDa and 23 kDa, respectively. The isoelectric points of the enzymes were 8.6 for xylanase A and 5.9 for xylanase B (not shown).

Table 3
Relative activities of xylanases A and B towards different substrates

Substrate	Xylanase A [a] (%)	Xylanase B [b] (%)
Birchwood xylan	100	100
Oat spelts xylan	335	180
Wheat straw hemicellulose A	297	170
Wheat straw hemicellulose B	268	189

[a] Activity of xylanase A: 966 U mg^{-1} prot.
[b] Activity of xylanase B: 199 U mg^{-1} prot.

3.4. Immunological properties

As shown in Fig. 3, antibodies against each xylanase react only with the homologous enzyme. No cross-reactivity was observed either with the purified enzymes or the isoenzymes present in the crude extract.

3.5. Effect of pH and temperature

Xylanase A has a pH optimum of 7.0 and xylanase B of 3.5 (Fig. 4). When preincubated for

Table 2
Summary of the purification of xylanase B.

Purification step	Volume (ml)	Proteins (mg ml^{-1})	Total activity (units)	Spec. activity (U mg^{-1} prot.)
Crude supernatant	500	0.47	9300	39.6
$(NH_4)_2SO_4$	8.0	13.5	6874	63.7
Bio-Gel P-100 (fraction B)	14.7	0.98	1783	122
DEAE-cellulose	21.6	0.34	1034	141
CM-Sephadex	40.0	0.05	398	199

24 h at 4° C in a pH range from 3 to 9, enzyme A retained almost all activity between pH 6 and 7.5, while xylanase B showed similar stability in the range of pH 4.5 to 5.5. Both enzymes were stable when incubated for 3 h at 40° C and lost about 40% of their activity when kept for 3 h at 60° C. Highest activity was obtained at 60° C for xylanase A and at 50° C for the B form.

3.6. Substrate specificity

The activity of the purified enzymes towards several substrates is presented in Table 3. Both enzymes showed similar substrate specificity, and were more active on wheat straw hemicelluloses and oat spelts xylan than on birchwood xylan. Xylanase A, however, showed higher specific activities than xylanase B towards the various xylans

A:

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                5                  10                  15
Gly-Pro-X-Asp-Ile-Tyr-Ser-Ser-Gly-Gly-Thr-Pro-Pro-Val-Ala-Asn-His-
      (Cys)          (Glu-Ala)    (Asp)        (Cys)        (Ala)
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B:

Species	Sequence	Reference
P. purpurogenum	SINYVQNYNGNLGAFSYNEGAGTFS	This work
A. tubigensis	SAGINYVQNYNGNLGDFTYDESAGTFS	de Graaff et al. (1994)
A. awamori	same	Hessing et al. (1994)
T. terrestris	AAGINYVQNYNGNLGYFTYNEGAGQFS	Gilbert et al. (1992)
A. kawashii	SAGINYVQNYNGNLADFTYDESAGTFS	Ito et al. (1992)

Fig. 5. (A) Amino terminal sequence of *P. purpurogenum* xylanase A and α-L-arabinofuranosidase B from *A. niger* (Flippi et al., 1993). Residues in parentheses are those found in the arabinofuranosidase. (B) Amino terminal sequences of *P. purpurogenum* xylanase B and other homologous fungal xylanases. Residues conserved in all sequences are highlighted.

assayed (compare the values in Tables 1 and 2 for the purified enzymes).

3.7. Effect of possible inhibitors on enzyme activity

The effect of several substances on the activity of the purified xylanases was assayed at a final concentration of 1 mM. $MnCl_2$, $CoCl_2$, and $CaCl_2$ had no effect; $BaCl_2$, $CuSO_4$, EDTA and iodoacetate inhibited about 30–50%. Complete inactivation of both enzymes was observed with *N*-bromosuccinimide (suggesting the presence of essential tryptophan residues) and SDS, and to a lesser extent (about 60%) with Hg^{2+}.

3.8. Amino acid sequence

The amino acid sequence of the amino terminal ends of xylanases A and B are presented in Fig. 5. The enzymes showed no significant homology in this part of the sequence. A search for homologies to other proteins was made through the Protein Information Resource Network (Georgetown University, Washington DC, USA). Highest homology for xylanase A was found with α-L arabinofuranosidase B from *Aspergillus niger* (Flippi et al., 1993) (Fig. 5A). Homologies higher than 75% for the N terminal segment of xylanase B were detected with a xylanase from *A. tubigensis* (de Graaff et al., 1994), xylanase A from *A. awamori* (Hessing et al., 1994), xylanase II from *Thielavia terrestris* (Gilbert et al., 1992) and xylanase C from *A. kawachii* (Ito et al., 1992) (Fig. 5B).

4. Discussion

Work on xylanases from soft-rot fungi has been performed especially with species from the genera *Trichoderma* and *Aspergillus* (Wong et al., 1988). *Penicillium* xylanases are known from *P. janthinellum* (Takenishi and Tsujisaka, 1973; Milagres et al., 1993), *P. funiculosum* (Mishra et al., 1985), *P. herquei* (Funaguma et al., 1991), *P. chrysogenum* (Haas et al., 1992) and *P. capsulatum* (Filho et al., 1992). Comparative properties of *Penicillium* xylanases are shown in Table 4. The enzymes have a small size (MW ≤ 35 000) and are mostly acidic. The inhibition by *N*-bromosuccinimide and Hg ions observed in this work has also been found in other *Penicillium* xylanases (Haas et al., 1992; Filho et al., 1992).

The presence of more than one form of xylanases in culture filtrates has been detected in cultures of various fungi (Wong et al., 1988). The occurrence of different enzyme forms may be the result of proteolysis in the culture medium, post-translational modifications (glycosylation) or they may be the product of separate genes. The two xylanases described in this work show considerable differences in their properties. Of particular relevance are the fact that their antisera do not cross-react and the lack of homology in their

Table 4
Properties of xylanases purified from culture filtrates of different *Penicillium* species

Species	Ref.	Form	Mol. mass (kDa)	p*I*	Optimum	
					pH	T (°C)
P. purpurogenum	this work	A	33	8.6	7	60
		B	23	5.9	3.5	50
P. chrysogenum	Haas et al. (1992)		35	4.2	5–7	40
P. janthinellum	Takenishi and Tsujisaka (1973)				5.3	
P. herquei	Funaguma et al. (1991)				3	50
P. capsulatum	Filho et al. (1992)	A	28.5	5–5.2	4	47–48
		B	29	5–5.2	4	47–48

amino terminal sequences. These results strongly suggest that we are dealing with different proteins, products of separate genes. Definite proof of this hypothesis will be obtained by isolation and sequencing of their genes. Work in this regard is currently in progress.

Xylan is a complex molecule and several enzymes are required for its hydrolysis. If the xylanases found are different enzymes, it is tempting to suggest that they may have different bond-breaking specificity and therefore may be acting cooperatively in xylan degradation. Preliminary data (not shown) suggest a difference in the product pattern obtained by both enzymes. The structural complexity of xylan makes it difficult to determine the precise specificity of the xylanases (Maringer et al., 1995), but the use of model substrates (xylooligosaccharides) may help clarify this matter.

Cellulases and xylanases, based on their amino acid sequence and on hydrophobic cluster analysis, have been classified in a number of families (Gilkes et al., 1991; Henrissat and Bairoch, 1993). Xylanase B can probably be assigned to family G based on its high homology to several xylanases of this family (Fig. 5B). No family assignment can be made for xylanase A at this point, since the only homology found is to arabinofuranosidase B of *A. niger* (de Graaff et al., 1994). It is interesting to note that neither xylanase shows significant homology to the xylanase produced by *P. chrysogenum* (Haas et al., 1992).

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