Xylanases from *Streptomyces cyaneus*: their production, purification and characterization

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(Received 20 August 1992; revised 26 April 1993; accepted 7 May 1993)

Streptomyces cyaneus produces extracellular xylanases in good yield when grown on ball-milled straw and xylan. Ball-milled straw induced nearly twice as much of these activities as did xylan, but phenolic substances released from the straw impeded purification of enzymes. Three extracellular xylanases, I, II and III, were identified in xylan-grown culture supernatants. Xylanases I and II were each purified to give single bands by SDS-PAGE. They had M_r values of 37 500 and 34 000 respectively, while that of III was apparently 45 000. pI values were 5.1 (I) and 5.3 (II) while pH and temperature optima, in 10 min assays, were 8.5, 72°C (I) and 6.5, 65°C (II).

Introduction

We have studied the abilities of three actinomycetes, *Actinomadura* sp., *Thermomonospora mesophila* and *Streptomyces cyaneus*, to solubilize lignocellulose (Mason, 1988; Zimmermann & Broda, 1989; Broda *et al.*, 1989). Of the three strains, *S. cyaneus* had the best growth characteristics, and it produced [^{14}C -lignin]-lignocellulose solubilization activity earlier and in greater yield than did the other two strains (Mason *et al.*, 1988).

In plants, lignin, cellulose and hemicellulose are closely associated; hemicelluloses (especially xylans) and lignin are cross-linked by various covalent bonds (Timell, 1967). Hydrolysis of xylan facilitates the removal of lignin from biomass. Thus xylan-degrading enzymes are increasingly being used in biobleaching of pulps for paper manufacture (Buchert *et al.*, 1992). For this reason we began an investigation of xylan-degradation by *S. cyaneus*.

When the organism is grown on ball-milled straw, substantial amounts of acid-precipitable (phenolic) materials are produced, making the isolation of extracellular proteins so difficult that the cellulase, xylanase and [^{14}C]lignocellulose solubilization activities cannot readily be characterized (Mason *et al.*, 1988). The experiments described here define better conditions for the production and isolation of proteins involved in xylan conversion.

Methods

Organism, media and culture conditions. *Streptomyces cyaneus* MT813 (McCarthy & Broda, 1984; Mason *et al.*, 1988) was cultured in a medium containing (l^{-1}) 0.1 g $(NH_4)_2SO_4$, 0.3 g NaCl, 0.1 g $MgSO_4$, 0.2 g $CaCO_3$, 1 g Bacto yeast extract, and 1 ml trace elements (0.02% $FeSO_4 \cdot 7H_2O$, 0.018% $ZnSO_4 \cdot 7H_2O$ and 0.002% $MnSO_4 \cdot 4H_2O$). The medium was adjusted to pH 7.0 with Na_2HPO_4 . The carbon sources listed in Table 1 were used at final concentrations of 0.2%. Cultures were inoculated with spore suspensions and incubated at 37°C with shaking at 150 r.p.m.

Preparation of ball-milled straw. Chopped cereal straw and ball-bearings at 1:1 (v/v) were packed into the ball-milling chamber of a Sieberttechnik ball-mill and milled for 72 h at room temperature. The ground material was sieved through a 60 mesh sieve to remove large particles before use.

Bicinchoninate reagent. Reducing sugar concentrations were assayed using the bicinchoninate/ Cu^{2+} reagent and a procedure adapted from Mopper & Gindler (1973) and Sinner & Puls (1978). The reagent was prepared as follows. Solution A contained disodium 2,2'-bicinchoninate (0.65 g), sodium carbonate (anhydrous; 0.36 g), distilled water to 500 ml. Solution B contained aspartic acid (3.7 g), sodium carbonate (anhydrous; 5.0 g), copper(II) sulphate (anhydrous; 1 g), distilled water to 150 ml. Copper(II) sulphate was first dissolved in 40 ml water and then mixed with the solution of aspartic acid and sodium carbonate. Before use, A and B were combined in a ratio of 23:1 (v/v).

Preparation of xylan suspension. Remazol brilliant blue xylan (RBB-xylan) was prepared according to Biely *et al.* (1985). Oatspelt arabinoglucuronoxylan (Sigma) suspension was prepared as described by Wang & Broda (1992); this involved two centrifugations, one to remove larger particles and the other to remove soluble xylan. For routine assays suspensions contained 4 mg xylan ml^{-1} . Arabino-galactan (larchwood, Sigma), a polysaccharide with a β -1,3-linked main chain, was prepared in the same way.

Enzyme assays. Xylan-degrading activity was measured by a procedure adapted from Zimmermann *et al.* (1988). Enzyme (25 μ l)

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was mixed with 75 μ l of the xylan (4 mg ml⁻¹) suspension prepared as described above in barbital buffer (25 mM) pH 7.8 and the mixture was incubated at 60 °C for 10 min. At the end of the incubation the mixture was cooled quickly in an ice-water bath. Bicinchoninate/Cu²⁺ reagent (1 ml) was added to the reaction mixture and incubated at 80 °C for 10 min. The reaction was cooled again in an ice-water bath and the absorbance at 562 nm was determined. Appropriate controls were included in all assays. All assays were carried out in 1.5 ml microcentrifuge tubes. One unit (U) of xylanase is defined as the amount of enzyme that releases 1 μ mol of xylose equivalent min⁻¹ under the above conditions.

β -Glycosidase activity was measured by determining the amount of *p*-nitrophenol (pNP) or *o*-nitrophenol (oNP) released from the appropriate substrates. Substrates were dissolved in distilled water at concentrations of 10 mM. The reaction mixture contained 50 μ l of enzyme preparation, 50 μ l of substrate and 50 μ l of 50 mM-sodium barbital buffer, pH 7.8. After incubation at 37 °C, 1 ml 1 M-Na₂CO₃ was added to the enzyme reaction mixture and the absorbance at 400 nm (for pNP) or 420 nm (for oNP) was determined immediately.

Hydrolysis of arabinogalactan was assayed by incubating 25 μ l of the enzyme preparation with 75 μ l of an arabinogalactan suspension (4 mg ml⁻¹) in 25 mM-sodium barbital buffer, pH 7.8 at 37 °C overnight. Reducing sugar produced was measured with the bicinchoninate/Cu²⁺ reagent.

Cellulase activity was assayed by incubating 25 μ l of enzyme with 75 μ l of a 2% (w/v) suspension of microcrystalline cellulose in 10 mM-maleate, pH 5.4, at 37 °C overnight. After incubation the undigested substrate was removed by centrifugation at 12000 *g* for 5 min. The reducing sugar content of the supernatant was measured by the bicinchoninate/Cu²⁺ method.

Hydrolysis of RBB-xylan was determined by the release of dye to the supernatant following precipitation of undigested substrate by different amounts of 2 M-NaCl (unpublished results).

Determination of protein concentration. Protein concentration was measured by the method of Bradford (1976). A sample (800 μ l) of protein containing 1–20 μ g protein was mixed with 200 μ l of dye (Bio-Rad) and allowed to stand at room temperature for 5 min. The absorbance at 595 nm was determined and protein concentration was calculated by reference to a standard curve using suitable dilutions of bovine serum albumin (100 μ g ml⁻¹; Sigma).

SDS-PAGE. Separation of proteins by SDS-PAGE was as described by Laemmli (1970). *M_r* values were determined by comparing the electrophoretic mobility of the unknown proteins with those of standards (Sigma). Gels were stained overnight at room temperature with Coomassie Brilliant Blue.

Stain for xylanase activity. After electrophoresis, the SDS gel was soaked in 20 mM-Tris/HCl, pH 7.5, containing 0.7% Triton X-100 with gentle shaking for 1 h. The procedure was repeated twice. Gels were then incubated with 20 mM-Tris/HCl, pH 7.5, for 20 min, washed with distilled water, and incubated with a 0.1% xylan suspension at 37 °C for 2 h. The gel was washed again with distilled water, stained with Congo Red (0.5 mg ml⁻¹) for 15 min, and then destained in 1 M-NaCl for at least 15 min. Xylanase bands were yellow and the background was red. The gel was photographed within a few hours or was treated with 1 M-HCl for longer storage. After acidification the xylanase bands were white against a blue background.

Isoelectric focusing. Isoelectric focusing was performed in 5% (w/v) polyacrylamide gels (150 × 180 × 0.75 mm). Gels were stained for protein as above.

Preparation of dialysate for purifying xylanases. Protein in the culture filtrate was precipitated by addition of (NH₄)₂SO₄ to 60% saturation.

The precipitate was dissolved in 20 mM-Tris/HCl buffer, pH 7.5, and dialysed overnight against 20 mM-sodium acetate, pH 5.0. The dialysate was centrifuged in a bench-top centrifuge. The supernatant was used for isolation of xylanase II and the precipitate was dissolved in 20 mM-Tris/HCl, pH 7.5 for isolation of xylanase I.

Purification of xylanase I. QAE-Sephadex pre-equilibrated in 20 mM-Tris/HCl, pH 7.5, was added to the dialysate to a final concentration of 1 mg ml⁻¹. The QAE-Sephadex (where the bulk of the phenolic pigments adsorbed) was filtered away and the filtrate was then loaded on a DEAE-Sephacrose column (1.5 × 15 cm). The column was equilibrated with 20 mM-Tris/HCl, pH 7.5, and protein was eluted using 100 ml of a linear gradient (0–0.65 M) of Na₂SO₄ in the same buffer.

Purification of xylanase II. The protein precipitated with (NH₄)₂SO₄ was dissolved in and dialysed against 50 mM-acetate buffer, pH 5.0, then loaded on a DEAE-Sephacrose column (1.6 × 35 cm). Protein was eluted with a gradient from 0% to 65% of 1 M-NaCl in 50 mM-acetate buffer, pH 5.0. Fractions containing the major xylanase activity (fractions 4 to 14) were collected and de-salted using a column of Sephadex G25.

The pooled partially purified xylanase samples eluted from DEAE-Sephacrose were freeze-dried and loaded on a MonoQ column. The column was eluted with an NaCl gradient (0% to 65% of a 1 M-NaCl solution in 20 ml 20 mM-Tris/HCl, pH 7.5).

Results

Effect of carbon source

The yields of xylanase activity on various carbon sources were compared with that given by growth on ball-milled straw. Neither monosaccharides nor disaccharides induced activity, while each of the polysaccharides did (Table 1). Of the latter, xylan was the best. Because of the difficulties associated with production of a pigment when ball-milled straw was used as substrate, xylan-grown culture filtrates were used in all subsequent investigations.

Production of xylanase activity was 2–3-fold higher in ball-milled straw than in xylan media. However, cul-

Table 1. *Effect of carbon source on the production of xylanase activities*

Carbon source	Mean xylanase activity (mU ml ⁻¹)
CM cellulose	29
Cellulose MN-300	39
Soluble starch	29
Oatspelt xylan	145 (3)
Ball-milled straw	269 (3)

D-Glucose, D-xylose, lactose, maltose and cellobiose all gave values of < 0.001. Data labelled (3) are the means from triplicate experiments; other data are the means of duplicates. All the samples were taken at 96 h.

Fig. 1. Variation in xylanase activities produced by *S. cyaneus* grown for 10 d in a medium containing either 0.2% xylan (○) or 0.2% BMS (□) with shaking at 37 °C.

tivation in either medium was characterized by marked peaking and troughing of activity (Fig. 1). The phenomenon has been noted before in xylanase production by *Talaromyces emersonii* (Tuohy & Coughlan, 1992). There the peaking and troughing was attributed to differences in the timing of induction of separate components of the xylanase system – the products of action of one component inducing the synthesis of another, and so on – as well as to differential inactivation by proteases, or variation in the pH during uncontrolled cultivation conditions. The growth conditions reported here were not pH-controlled but the pH rose only from an initial 7.0 to 7.8 at harvest.

Samples of culture filtrates clarified agar gels containing 1% oatspelt xylan suspension. They also catalysed a rapid decrease in the turbidity of a 1% oatspelt xylan suspension (Wang & Broda, 1992). Moreover, such samples released dye from RBB-xylan. These findings indicate that the filtrates of xylan-grown cultures contain endo- β -1,4-xylanase activity, i.e. they catalyse hydrolysis of the oatspelt arabinoxylan backbone (Biely *et al.*, 1985).

Effect of pH, temperature and salts on xylanase activity

Xylanase activity in culture filtrates, as measured by the release of reducing sugars from suspensions of oatspelt arabinoxylan, was detected over a broad pH range (3–9) but was optimal at pH 7.8 in 10 min assays. Reducing

Fig. 2. Distribution of xylan-degrading activity in 0.5 ml fractions obtained by gel filtration on Superose 12 of extracellular proteins produced by *S. cyaneus* grown in a medium containing xylan.

sugar production from this substrate by crude filtrates was detected at temperatures ranging from 20–80 °C and was optimal at 60 °C.

Xylanase activities in crude filtrates in barbital or phosphate buffers were higher than that in Tris/HCl buffer. Routine assays of such activity in subsequent investigations were therefore carried out at 60 °C in 25 mM-barbital buffer, pH 7.8.

Separation of xylanase activities by gel filtration and SDS-PAGE

Filtrates of 4-d-old xylan-grown cultures were subjected to FPLC gel filtration on Superose 12. Major peaks of activity (peaks I, II and III) were found in fractions 16, 27 and 34 (Fig. 2). The materials in these fractions were analysed using the zymogram technique for xylanase activity. The corresponding M_r values were calculated to be 37 500, 34 000 and 45 000 respectively. The fact that the three enzyme activities were not eluted in order of decreasing M_r value, as ideally they would be, may reflect interaction with the column matrix.

Purification of xylanase I

By a four-step procedure, FPLC gel filtration, precipitation, QAE-Sephadex adsorption and anion exchange chromatography (Fig. 3a), 430 μ g of xylanase I was obtained from 1700 ml of culture filtrate. By SDS-PAGE this preparation gave only one visible band with an M_r of 37 500; a zymogram showed that the M_r 37 500 protein was the only xylan-solubilizing activity in that preparation (Fig. 3b). Efficient degradation of xylan is effected by the synergistic interaction of side-chains and main-chain hydrolysing activities. Fractionation of such components would disrupt the potential for such synergy.

Fig. 3. (a) Separation of xylanase I activity (○) by anion exchange chromatography on DEAE-Sepharose. The column had a bed volume of 35 ml. Approximately 4.4 mg protein was loaded, then eluted with a sodium sulphate gradient (—) in 20 mM-Tris/HCl, pH 7.5. Fraction size: 2 ml. Flow rate: 1 ml min⁻¹. (b) SDS-PAGE of xylanase I. A 15 µg sample of a preparation obtained after anion exchange chromatography on DEAE-Sepharose was loaded on the gel. Lane 1, M_r markers; lane 2, xylanase I.

This would explain why the recovery of xylanase activity was low (Table 2). Nevertheless, the procedure gave an enzyme preparation without detectable contamination by other xylanases and suitable for further characterization.

Purification of xylanase II

Xylanase II was purified by a different four-step procedure involving filtration, precipitation and two cycles of anion exchange chromatography (Fig. 4 and Table 2). After the last step the enzyme preparation (fraction 9 to 13, Fig. 4b) contained only one protein by SDS-PAGE analysis: when the gel was stained for xylanase activity, a single band corresponding to that stained for protein was detected. The M_r was estimated to be 34000 (Fig. 4c).

The pI values of purified preparations of xylanases I and II were determined by isoelectric focusing to be 5.1 and 5.2 respectively. Xylanases I and II were optimally active at 72 °C and 65 °C, respectively. Xylanase I catalysed the liberation of reducing sugars from xylan at pH values ranging from 4.0 to 10.5. Activity was optimal

at pH 8.0 and was 80% of the optimum at pH 6.0 and at pH 10.5. Xylanase II showed activity at all pH values tested between 3.0 and 10; the optimum pH value was 6.5, and activity was 80% of optimum at pH 5.5 and 7.5 (Fig. 5).

Substrate specificity

Each of the purified proteins catalysed the release of dye from RBB-xylan. Thus, they are endoxylanases or exhibit endoxylanase activity, i.e. they cleave the β -1,4-linked backbone of oat spelt arabinoxylan. In long-term incubations (37 °C overnight) both proteins liberated reducing sugars from arabinoxylan and type II arabinogalactan, a β -1,3-linked polymer of β -D-galactopyranose with 6-linked β -D-galactopyranosyl and α -L-arabinofuranosyl initiated side-chains. Neither enzyme had detectable action against cellulose. Each protein could be differentiated by its actions against artificial substrates (Table 3). Xylanase I acted significantly against the pNP derivative of α -L-arabinofuranoside. By contrast, xylanase II lacked this activity. Xylanase II has activity against pNP derivatives of β -D-mannopyranoside but xylanase I lacks this activity. Further work, including identification

Fig. 4. (a) Separation of xylanase II activity (O) by anion exchange chromatography on DEAE-Sepharose. Approximately 0.5 mg protein was loaded on the column. The protein was eluted with an NaCl gradient (—) in 50 mM-sodium acetate, pH 5.0. Fraction size: 2 ml. Column bed volume: 35 ml. Flow rate: 1 ml min⁻¹. (b) Separation of xylanase II activity by anion exchange chromatography on a MonoQ column. About 0.5 mg protein was loaded. The protein was eluted with an NaCl gradient in 20 mM-Tris/HCl, pH 7.5. Fraction size: 0.5 ml. Flow rate: 1 ml min⁻¹. (c) SDS-PAGE of xylanase II. A 10 µg sample of a preparation obtained after anion exchange chromatography on MonoQ was loaded on the gel. Lane 1, xylanase II; lane 2, M_r markers.

Table 2. *Purification of xylan-degrading activities I and II*

		Activity I			Activity II		
	Filtrate	Precipitated with ammonium sulphate	QAE Sephadex adsorption	DEAE Sepharose	Precipitated with ammonium sulphate	DEAE Sepharose	MonoQ
Volume (ml)	1700	50	40	14	50	240	18
Xylanase activity (mU ml ⁻¹)	105	552	460	292	522	8	18.4
Total activity (mU)	178 500	27 600	18 400	4088	26 120	1920	331
Concentration of protein (mg ml ⁻¹)	75	208	112	30.4	61.4	4.4	8.3
Total protein (mg)	127	10.4	4.4	0.43	3.07	1.06	0.15
Specific activity [mU (mg protein) ⁻¹]	1400	2653	4182	9507	8502	1818	2207
Percentage recovery	100	15.4	10.3	2.3	14.6	1.1	0.2

Fig. 5. Effect of temperature and pH on activities of xylanase I (□) and xylanase II (○), given as a percentage of the optimal activities. The incubation time for the enzyme reactions was 10 min. (a) Effect of temperature; the incubation buffer was 0.025 M-sodium barbital, pH 7.8. (b) Effect of pH. Three buffers were used for the enzyme activity assays: 0.025 M-sodium citrate, pH 3.0, 4.0, 5.0, 6.0; 0.025 M-sodium barbital, pH 7.0, 8.0; and 0.025 M-glycine/sodium hydroxide, pH 9.0, 10.4.

Table 3. Relative activities of xylanases

All values are means of duplicate assays with variations of less than 5%. The percentages of the amounts of products produced were calculated by using xylan ('natural' substrates; upper part of table) or pNP- β -D-xylopyranoside ('artificial' substrates; lower part of table) as standard.

Substrate	Xylanase I (%)	Xylanase II (%)
Xylan	100	100
Arabinogalactan	17.4	9.6
Cellulose-azure	0	0
pNP- β -D-xylopyranoside	100	100
pNP- β -D-galactopyranoside	0.82	23.2
pNP- β -D-mannopyranoside	0	69.8
pNP- β -D-glucopyranoside	0.82	0
pNP-L-arabinofuranoside	23.4	0

of the products of xylan degradation, will be required to determine the exact nature of the two enzymes.

Discussion

Streptomyces cyaneus was originally screened for its higher activity in degradation of lignin (McCarthy & Broda, 1984). However, lignin is tightly combined with hemicellulose and cellulose so that micro-organisms are faced with a lignocellulose complex rather than a simple carbon source. Compared with organisms such as *Bacillus* and yeast, *Streptomyces cyaneus* is well-equipped for degradation of the lignocellulose complex.

With the eight substrates tested, the two xylanases that were purified showed different patterns of substrate specificity. Although the main activity of each was against xylan, both xylanases show activity to arabinogalactan. Furthermore, xylanase I showed very high activity against pNP- β -D-xylopyranoside and significant activity against pNP- α -L-arabinofuranoside; xylanase II had lower activity against pNP- β -D-xylopyranoside and pNP- β -D-mannopyranoside and showed no activity against pNP- α -L-arabinofuranoside.

Due to the complex pattern of the enzymes produced by *S. cyaneus* and the difficulties in purifying these proteins, contamination by other proteins cannot be completely ruled out; however, the purified proteins gave single bands on SDS-gels. Multifunctionality of enzymes is another possibility in xylan degradation; this could have a role in removing side-chains from the complex natural substrate, where different residues are widely present, such as in arabinoxylan. Some xylanases are reported to be arabinose-liberating (Matte & Forsberg, 1992; Grabski & Jeffries, 1991), while other xylanases do not liberate arabinose from the main xylan chain (Dekker & Richards, 1976). Thus, the hydrolysis of arabinogalactan in this case may reflect merely the slow release of arabinose therefrom, though further work would be required to establish this. Two xylanase genes have now been cloned and expressed in this laboratory. Characterization of the expressed xylanases in *E. coli* will allow clarification of their substrate specificities and this work will be reported later.

This work was part of a programme supported jointly by the Agricultural and Food Research Council and Venture Research International. P. Wang thanks the Chinese Academy of Science for a scholarship. We thank A. J. McCarthy and the late M. P. Coughlan for their comments.

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