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

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*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 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 [14C-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 [14C]lignocellulose solubilization activities cannot be readily 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 (I·) 0·1 g (NH₄)₂SO₄, 0·3 g NaCl, 0·1 g MgSO₄, 0·2 g CaCO₃, 1 g Bacto yeast extract, and 1 ml trace elements (0·02% FeSO₄.7H₂O, 0·018% ZnSO₄.7H₂O and 0·002% MnSO₄.4H₂O). The medium was adjusted to pH 7·0 with Na₃PO₄. 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 barley straw were packed into the ball-milling chamber of a Siebertechnik 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/Cu²⁺ 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 (37 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 Bely et al. (1985). Oatspelt arabinoxylan (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. Arabinogalactan (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 µl)
was mixed with 75 µl 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 described above in barbital buffer (25 mM) concentrations of 10 mM. The reaction mixture contained 50 µl of enzyme preparation, 50 µl of substrate and 50 µl 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 595 nm was determined. 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 µmol 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 µl of enzyme preparation, 50 µl of substrate and 50 µl 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 µl of the enzyme preparation with 75 µl 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 µl of enzyme with 75 µl 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 µl) of protein containing 1-20 µg protein was mixed with 200 µl of dye (Bio-Rad) and allowed to stand at room temperature for 5 min. The absorbance at 595 nm was measured and protein concentration was calculated by reference to a standard curve using suitable dilutions of bovine serum albumin (100 µg ml⁻¹; Sigma).

**SDS-PAGE.** Separation of proteins by SDS-PAGE was as described by Laemmli (1970). M₁ 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-Sephadex 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-Sephadex 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-Sephadex 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) |

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.
activation 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 (Biciy *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 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 μg 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.
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 Mr 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 oatspelt arabininoxylan. 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...
**Table 2. Purification of xylan-degrading activities I and II**

<table>
<thead>
<tr>
<th></th>
<th>Activity I</th>
<th></th>
<th>Activity II</th>
<th></th>
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<tr>
<td></td>
<td>Filtrate</td>
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<td>QAE</td>
<td>DEAE</td>
<td>Precipitated</td>
<td>DEAE</td>
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<td></td>
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<td>adsorption</td>
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<td>14</td>
<td>50</td>
<td>240</td>
</tr>
<tr>
<td>Xylanase activity</td>
<td>105</td>
<td>552</td>
<td>460</td>
<td>292</td>
<td>522</td>
<td>8</td>
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<tr>
<td>(mU ml⁻¹)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total activity (mU)</td>
<td>178 500</td>
<td>27 600</td>
<td>18 400</td>
<td>4088</td>
<td>26 120</td>
<td>1920</td>
</tr>
<tr>
<td>Concentration of protein (mg ml⁻¹)</td>
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<td>208</td>
<td>112</td>
<td>304</td>
<td>61.4</td>
<td>44</td>
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<tr>
<td>Total protein (mg)</td>
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<td>10.4</td>
<td>4.4</td>
<td>0.43</td>
<td>3.07</td>
<td>1.06</td>
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<tr>
<td>Specific activity</td>
<td>1400</td>
<td>2653</td>
<td>4182</td>
<td>9 507</td>
<td>8 502</td>
<td>18 18</td>
</tr>
<tr>
<td>[mU (mg protein)⁻¹]</td>
<td></td>
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<td>Percentage recovery</td>
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<td>15.4</td>
<td>10.3</td>
<td>2.3</td>
<td>146</td>
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</tr>
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Fig. 4. (a) Separation of xylanase II activity (○) 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, M₇ markers.
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 arabino-
galactan. 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 arabino-
galactan 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. Character-
ization of the expressed xylanases in *E. coli* will allow
clarification of their substrate specificities and this work
will be reported later.

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