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A Simple Procedure for the Large-scale Purification of β-D-xylanase from *Trichoderma viride*

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SUMMARY

A simple procedure is described for the purification of gram quantities of β-D-xylanase from a commercially available Trichoderma viride *culture filtrate. Chromatography of the crude extract on CM-Sepharose CL-6B gave two partially separated peaks of β-D-xylanase activity, and for convenience these have been termed xylanases I and II. Each has cellulase activity. The cellulase and xylanase activities were not separated by further purification on Ultrogel AcA 54 or Phenyl Sepharose CL-4B. Each of these xylanases was purified essentially to homogeneity (by the criterion of isoelectric focusing) and was free of protease, amylase and glycosidase activities. Physical and kinetic properties of xylanases I and II were identical, indicating that the separation of CM-Sepharose CL-6B may simply have been an artefact of chromatography. However, this pattern was reproducible, being obtained on several occasions. Each enzyme separated into two protein bands on isoelectric focusing. The major band had a pI of 8·45 and a very minor component had a pI of 7·3. Optimal activity was at pH 4·5 and 50 °C and the enzymes were stable over a pH range of 3·4–7·9 and at temperatures below 55 °C. Apparent K_ms were 3·33 mg ml^{-1} on rye flour arabinoxylan and 1·33 mg ml^{-1} on larch wood xylan. The enzymes partially hydrolysed larch wood xylan to oligosaccharides with two or three D-xylosyl residues. Rye flour arabinoxylan was hydrolysed to high molecular weight oligosaccharides which were not fractionated on Bio-Gel P-2.*

INTRODUCTION

Xylanases ((1→4)-β-D-xylan xylanohydrolase, EC 3.2.1.8) catalyse the *endo*-depolymerisation of (1→4)-β-D-xylans and of partially substituted

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D-xylans, i.e. arabinoxylans (pentosans) and 4-*O*-methyl glucuronoxylans (Matheson & McCleary, 1985). Xylanases are produced by a wide range of fungi (Dekker & Richards, 1976) and a number have been purified and characterised (Dekker & Richards, 1976; Baker *et al.*, 1977; Paice *et al.*, 1978; Biely *et al.*, 1981; Yoshioka *et al.*, 1981; Comtat, 1983; Biely & Vršanská, 1983; Labavitch & Greve, 1983; Hoebler & Brillouet, 1984; Frederick *et al.*, 1985; Tan *et al.*, 1985).

Interest in xylanases originates from their key role in the degradation of (1→4)-β-D-xylans and related polysaccharides, which are a major constituent of ligno-cellulosic waste materials such as straw, bagasse and grain husks (Timell, 1962; Wilkie, 1978). Because xylans and substituted xylans are major components of plant cell walls (Aspinall, 1981), xylanases are also thought to aid in the pathogen infection of plants (Baker *et al.*, 1977) and the fungal degradation of wood (Timell, 1965).

The major endosperm cell wall polysaccharide of wheat and rye is arabinoxylan, which consists of a (1→4)-β-D-xylan backbone with single L-arabinosyl groups which are alpha-linked through *O*-3 (or to a lesser extent through *O*-2) to a proportion of the D-xylosyl residues (Montgomery & Smith, 1956). The fine structures of arabinoxylans and D-xylans have been studied by chemical (Ewald & Perlin, 1959; Medcalf *et al.*, 1968; Brillouet *et al.*, 1982) and enzymic methods (Goldschmid & Perlin, 1963; Comtat & Joseleau, 1981; Kusekabe *et al.*, 1983). The degree of substitution on the D-xylan backbone affects the degree of hydrolysis by β-xylanase. Hydrolysis of lightly substituted D-xylans is extensive and yields low molecular weight oligosaccharide products which can be readily characterised. Highly substituted D-xylans such as wheat and rye flour arabinoxylans are only partially hydrolysed, yielding high molecular weight oligosaccharides which are difficult to characterise. With these polysaccharides, chemical procedures would appear to be preferable for structural studies. However, we have found β-xylanase valuable in defining the functional contribution of wheat flour arabinoxylans to dough development and to various bread properties such as loaf volume and crumb and crust structure (McCleary *et al.*, 1986). Earlier enzymic studies (Tracey, 1964; Wrench, 1965) with crude snail digestive juice were inconclusive due to the low xylanase activity present and to contamination by other enzymes.

To study the effect of the *in situ* depolymerisation of pentosans in wheat flour doughs by β-xylanase, it was essential to locate a source of this activity, and develop a simple purification format for the preparation of large quantities of this enzyme, free of contaminating protease and amylase activities. In this paper we describe a purification format for the preparation of gram quantities of essentially homogene-

ous (isoelectric focusing) enzyme in five days from Bioxylanase, a commercial *Trichoderma viride* preparation.

MATERIALS AND METHODS

Chemicals

All chemicals used were analytical grade unless specified otherwise. The enzyme source was Bioxylanase (a preparation from cultures of *Trichoderma viride*) which was a gift from Biocon (Australia) Pty Ltd, Melbourne. CM-Sepharose CL-6B, Sephadex G25 (superfine), and Phenyl-Sepharose CL-4B were from Pharmacia (South Seas) Pty Ltd, Sydney, Australia. Ultrogel AcA 54 was from LKB Produkter, Sweden. Bio-Gel P-2 was from Bio-Rad Laboratories Pty Ltd, Sydney, Australia.

Polysaccharides

Larch wood xylan was from Sigma Chemical Co., St Louis, Missouri, USA (Cat. No. X-3875). A sample (20 g) was dissolved in 400 ml of 2·5 M sodium hydroxide, neutralised with 50% v/v aqueous acetic acid and then treated with 10 ml of tris–acetate buffer (2 M, pH 6.9), 2 ml of calcium chloride (200 mM) and 50 μL of porcine pancreatic α-amylase (Sigma Cat. No. A4286, 780 units) at 40°C for 2 h. The solution was treated with two volumes of ethanol and the precipitated xylan was recovered by centrifugation, washed with ethanol and acetone, and dried under vacuum.

Barley flour (1→3)(1→4)β-D-glucan was purified as described previously (McCleary, 1986). Soluble starch was from May and Baker Ltd, Dagenham, England, and carboxymethyl (CM)-cellulose 4M6F and 7LF from Hercules Inc, Wilmington, Delaware, USA. Carob (*Ceratonia siliqua*) galactomannan and konjac (*Amorphophallus konjac*) glucomannan were prepared as described previously (McCleary *et al.*, 1983). Deacetylated glucomannan was prepared by treating native glucomannan with 1 M sodium hydroxide at room temperature for 1 h. The slurry was neutralised with 2 M hydrochloric acid and then dialysed against distilled water. Hydrocellulose was prepared by the treatment of cellulose powder with fuming hydrochloric acid followed by neutralisation (Miller, 1963). Tamarind amyloid (galactoxyloglucan) was purified from the seed of *Tamarindus indica* as described by Kooiman (1961). Nitrophenyl glycosides were purchased from Sigma. Hide powder azure was from Calbiochem.-Behring Aust. Pty Ltd, Sydney, Australia.

Rye flour arabinoxylan

Rye flour (200 g) was extracted in a Soxhlet apparatus with 1·5 litres of 80% (v/v) aqueous ethanol for 6 h. The residue was transferred to a sintered-glass funnel and excess liquid was removed by vacuum. The residue was added, with stirring, to 700 ml of distilled water, stirred for 1 h at 40°C and then centrifuged (3000 *g*, 30 min). The supernatant was recentrifuged (8000 *g* for 20 min), overlain with a few drops of toluene and stored at 4°C. The pellet was resuspended in 600 ml of distilled water and stirred for 30 min at 40°C. After centrifugation (8000 *g*, 20 min), the supernatant was stored and the pellet extracted once again. The three supernatant solutions were separately incubated at 80°C for 5 min and then cooled to 40°C. The solutions were adjusted to pH 6·9 with tris–acetate buffer (50 mM final concentration) and calcium chloride was added to a final concentration of 1 mM Ca^{2+}. Porcine pancreatic α-amylase (Sigma Cat. No. A4268, 200 U) was added to each extract, which was incubated at 40°C for 20 h in sealed containers, in the presence of a few drops of toluene. The solutions were then heated at 100°C for 10 min and centrifuged (10000 *g*, 30 min). The supernatants were mixed with four volumes of ethanol and the resultant precipitates were recovered by centrifugation, washed with ethanol and acetone, and dried under vacuum. Yields of arabinoxylan were: first extraction, 1·56 g; second extraction 0·80 g; and third extraction, less than 0·1 g. Alditol acetates of arabinoxylan from the first and second extractions were analysed by gas–liquid chromatography (Blakeney *et al.*, 1983) after hydrolysis with 2N trifluoroacetic acid (Albersheim *et al.*, 1967). For both extracts, arabinose plus xylose represented approximately 91% of total sugars. Other sugars present were glucose (~ 5%), mannose (~ 3%) and galactose (~ 1·5%). The ratio of arabinose to xylose was 42:58. Most of the D-glucose was removed by treatment for 2 h at 40°C and pH 4·5 with glucoamylase (Seikagaku Fir Biochemicals, Japan, 22 U mg^{-1}) at a ratio of 1 mg of enzyme to 200 mg of arabinoxylan. Treatment for 2 h at 40°C and pH 4·5 with guar seed β-mannanase (McCleary, 1983*a*; 20 U 200 mg^{-1} of pentosan) completely removed D-mannose and partially removed D-glucose (*ca* 20–30%), suggesting that a glucomannan and partially depolymerised starch were also present in the pentosan preparation.

Preparation of substrates

Rye flour arabinoxylan

To minimise the background level of reducing groups when rye flour arabinoxylan was used as substrate, a solution of the polymer (5 mg

ml^{-1}) was treated with sodium borohydride (500 mg 5 g^{-1} pentosan) for 60 min at room temperature. After neutralisation by the dropwise addition of 10% (v/v) acetic acid to remove excess borohydride, arabinoxylan was precipitated from solution by four volumes of ethanol, washed twice with ethanol and dried by solvent exchange with acetone. For the preparation of substrate solutions, the polymer (0·5 g) was moistened with ethanol, allowed to hydrate overnight in 40 ml distilled water at 4°C, and then heated to 80°C. The suspension was homogenised with an Ultraturrax and the concentration was adjusted to 10 mg ml^{-1} with distilled water. This solution was overlain with a few drops of toluene and stored in a sealed container at 4°C.

Larchwood xylan

Larchwood xylan (1 g) was dissolved in 20 ml of 1 M sodium hydroxide. The volume was adjusted to 150 ml with distilled water and the pH to 4.5 with 10% v/v acetic acid. The final volume was adjusted to 200 ml to produce a solution of 5 mg ml^{-1} xylan. The solution was then overlain with a few drops of toluene and stored in a sealed container at 4°C.

Enzyme assays

Xylanase

Enzyme preparations (50 μl) were incubated with rye flour pentosan (0·5 ml, 10 mg ml^{-1}) plus sodium acetate buffer (50 μl, 1 M, pH 4·5) or with 0·5 ml larch xylan (5 mg ml^{-1} in 100 mM sodium acetate buffer, pH 4·5), for 5 or 10 min at 30°C. The reactions were terminated by the addition of 0·5 ml of Somogyi copper reagent (Somogyi, 1952). Reducing sugars were then determined by a modified Somogyi method as described by McCleary & Glennie-Holmes (1985). One unit of enzyme activity is defined as the amount of enzyme required to release one micromole of xylose reducing sugar equivalents per minute at pH 4·5 and 30°C. A standard D-xylose solution (40 μg $assay^{-1}$) was analysed concurrently. Blank absorbance values for the substrate solutions increased with the age of the solution, probably because of a slow release of arabinosyl units off the xylan chain. Therefore, fresh solutions were prepared every two days.

Activities of xylanase on a range of other polysaccharide substrates were determined essentially by the same format as for xylan, but with the appropriate substrate. These included starch ((1→4)(1→6)-α-D-glucan), carob galactomannan ((1→4)-β-D-mannan with (1→6)-linked α-D-galactosyl residues), barley β-glucan ((1→3)(1→4)-β-D-glucan), hydrocellulose ((1→4)-β-D-glucan) and tamarind amyloid ((1→4)-β-D-glucan with (1→6)-linked α-D-xylosyl and (1→2)-linked β-D-galactosyl

residues). Glycosidase activities were determined by incubation of the enzyme (0·1 ml) with various *p*-nitrophenyl glycosides (0·1 ml, 10 mM) in 0·1 M acetate buffer (pH 4·5) for 0–5 min at 30°C. Reactions were terminated with 2% w/v sodium carbonate (3 ml) and the concentrations of released nitrophenol were determined by absorption at 410 nm (McCleary, 1983*b*). One unit of enzyme is defined as the amount of enzyme required to release one micromole of *p*-nitrophenol or reducing sugar equivalents per minute under defined conditions of temperature and pH.

Endo-*protease*

Enzyme preparations (0·2 ml) were incubated with hide powder azure (50 mg) in 2 ml of 0·1 M sodium acetate buffer (pH 4·5). The reactions were terminated by the addition of 2 ml of 10% trichloroacetic acid, the tubes centrifuged (10 min, 1000 *g*) and the absorbance at 590 nm measured. One unit of *endo*-protease activity is defined as the amount of enzyme which gives an absorbance increase of 1·0 at 590 nm min^{-1}, under the defined assay conditions.

Properties of purified xylanase

The optimal pH for activity was determined using the standard assay but with a range of citrate–phosphate buffers (50 mM citrate–100 mM phosphate, pH 3·3–7·7) (Dawson *et al.*, 1969) instead of 100 mM acetate buffer, pH 4·5. The pH stability of xylanase was determined by incubating aliquots of the xylanase preparations in 25 mM citrate–50 mM phosphate buffer (pH 3·4–7·9) at 4°C for 30 h. Residual activities of 50 μl aliquots were determined at pH 4·5 under standard assay conditions. The optimal temperature for activity was determined by assaying the enzyme over the temperature range of 30–65°C. In temperature stability studies, the enzyme was pre-incubated for 15 min over the same temperature range, equilibrated at 30°C, and residual activity was assayed under standard conditions. The effect of ions on activity was determined by incubating xylanase in 1 mM solutions (final concentration) of various ions for 15 min and then determining residual activity by incubating enzyme preparation (50 μl) with rye flour pentosan (0·5 ml, 10 mg ml^{-1}) plus sodium acetate buffer (50 μl, 1 M, pH 4·5) for 5 min at 30°C. Reducing sugars were determined using the Nelson–Somogyi reducing sugar assay (Somogyi, 1952). All metal salts used were chlorides, except for magnesium, which was the sulphate. EDTA was used as the disodium salt and cyanide was used as the potassium salt.

Molecular weights were determined by electrophoresis under denaturing conditions on 18% polyacrylamide gels (80 × 80 mm) with a

Pharmacia GE-2/4 apparatus. The gel buffer and running buffers were modified from Laemmli (1970) as described by Speirs & Brady (1981). Each xylanase track contained 10 μg of purified preparation. Molecular weight standards were supplied as the Pharmacia low molecular weight kit. Isoelectric points were determined over a pH range of 3·5–9·5 with an LKB Multiphor and commercial PAGE plates according to the manufacturer's instructions. (LKB Instruction Note No. 1804.)

Purification of β-xylanase from Bioxylanase preparation

Purification procedures were at 20°C unless otherwise specified. Fifty grams of Bioxylanase were dissolved in 500 ml of 20 mM sodium acetate buffer (pH 4) and the solution was readjusted to pH 4 with 10% acetic acid. After centrifugation (3000 *g*, 30 min) the supernatant was applied directly to a column (3·8 × 16 cm) of CM-Sepharose CL-6B. The column was washed with 1 litre of 20 mM acetate buffer (pH 4) and then with 3 litres of a linear potassium chloride gradient (0–0·5 M) in 20 mM acetate buffer (pH 4). A double peak of xylanase activity eluted at a potassium chloride concentration of 0·2–0·25 M. These peaks were collected separately and treated with ammonium sulphate (50 g 100 ml^{-1}) for 2 h. The pellets collected by centrifugation (12000 *g*, 10 min) were redissolved in a minimum volume of 20 mM sodium acetate buffer (pH 4) and applied separately to a column (4·3 × 90 cm) of Ultrogel AcA 54. The column was eluted with 20 mM acetate buffer (pH 4) and the active fractions were pooled and treated with sufficient solid ammonium sulphate to give a final concentration of 1 M. This solution was applied directly to a column (2·7 × 22 cm) of Phenyl Sepharose CL-4B and the column was eluted with 1·2 litres of a linear gradient of ammonium sulphate (1–0 M) plus ethylene glycol (0–50% (v/v)) in 10 mM sodium phosphate buffer (pH 7). The active fractions were pooled, desalted by chromatography on Sephadex G25 (superfine) and either stored frozen or treated with ammonium sulphate (50 g 100 ml^{-1}) and stored at 4°C.

Protein contents of column eluates and purified enzyme preparations were routinely determined by absorption at 280 nm. Precise protein determinations were performed as described by Lowry *et al.* (1951). The carbohydrate content of purified preparations was determined by phenol–sulphuric acid (Dubois *et al.*, 1956).

Analysis of hydrolysis products

Xylan solution (0·5 mg ml^{-1} in 100 ml of 10 mM sodium acetate buffer, pH 4·5) was incubated with 380 U xylanase for 24 h at 30°C. The solution was concentrated by rotary evaporation at 50°C to ~ 20 ml and

centrifuged (1000 *g*, 10 min). A 5 ml aliquot of the supernatant was applied to a Bio-Gel P-2 (< 400 mesh) column (2·5 × 80 cm) and eluted with distilled water at 60°C into 4 ml fractions. The total carbohydrate content of each fraction was analysed (Dubois *et al.*, 1956) and the carbohydrate peaks were retained and stored frozen. Alditol acetates of the glycoside constituents of each peak were analysed by gas–liquid chromatography (Blakeney *et al.*, 1983) after hydrolysis with 2N trifluoroacetic acid.

RESULTS AND DISCUSSION

Many fungal preparations yield two or more xylanase enzymes (e.g. Toda *et al.*, 1971; Comtat, 1983; Labavitch & Greve, 1983; Tan *et al.*, 1985). Bioxylanase, a preparation from cultures of *Trichoderma viride*, appeared to be no exception. Two xylanase fractions, termed xylanase I and xylanase II, were separated by chromatography on CM-Sepharose CL-6B (Fig. 1). Each fraction behaved identically on chromatography on Ultrogel AcA 54 (Fig. 2 — results for xylanase I only shown), Sephadex G25 (superfine) and on Phenyl Sepharose CL-6B (Fig. 3 — results for xylanase I only shown). Neither enzyme bound to DEAE-Sepharose CL-6B at pH values as high as 8 (in 20 mM tris–HCl). *Endo*-protease activity was completely removed by chromatography on Ultrogel AcA 54 (Fig. 2).

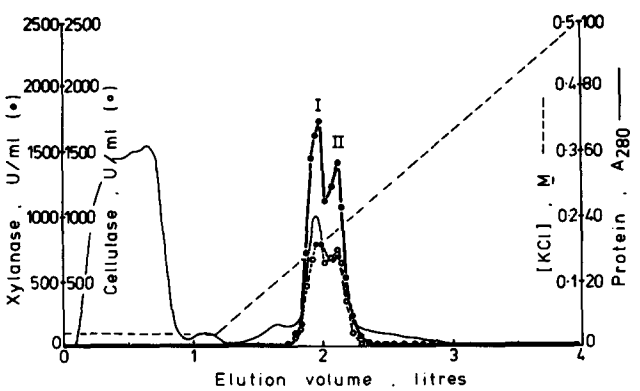

Fig. 1. Chromatography of Bioxylanase (50 g) on a CM-Sepharose CL-6B column (3·8 × 16 cm). The column was washed with 20 mM sodium acetate buffer (pH 4) and then eluted with a linear gradient (0–500 mM) of potassium chloride in 20 mM sodium acetate, pH 4.

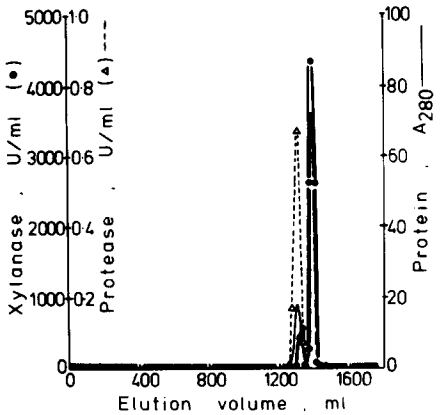

Fig. 2. Chromatography of xylanase I (from Bioxylanase) on an Ultrogel AcA 54 column (4·3 × 90 cm). The column was eluted with 20 mM sodium acetate buffer (pH 4). Protease activity units are defined as the absorbance increase at 590 nm ml^{-1} enzyme min^{-1} on hide powder azure under standard assay conditions.

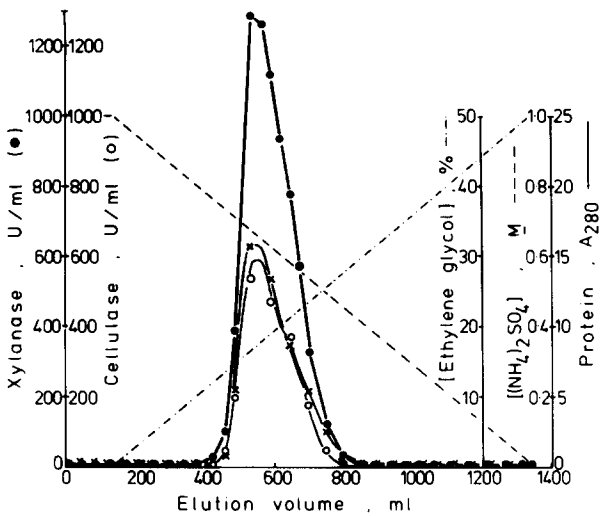

Fig. 3. Chromatography of xylanase I on a Phenyl Sepharose CL-6B column (2·7 × 22 cm). The column was eluted with a linear gradient of ammonium sulphate (1·0–0 M) plus ethylene glycol (0–50%) in 10 mM sodium phosphate buffer (pH 7).

The value of Bioxylanase as a source for the large scale preparation of xylanase is shown in Table 1. Each enzyme was purified essentially to homogeneity (by the criteria of isoelectric focusing and functionality) by three simple chromatographic steps, and the overall recovery of activity was 76·3%. This represents approximately 5 grams of enzyme

TABLE 1
Purification of Xylanase from Bioxylanase

Stage of purification	*Total protein (mg)*	*Total activity (U)*	*Specific activity ($U\ mg^{-1}$)*	*Yield (%)*	*Purification (-fold)*
Crude preparation	50000	402000	8·0	—	1
CM-Sepharose CL-6B I	5177	217150	41·9	54·0	5·2
II	3975	161230	40·6	40·1	5·1
Xylanase I					
Ultrogel AcA 54	3037	187730	61·8	46·7	7·7
Phenyl Sepharose	2766	171490	62·0	42·6	7·8
Xylanase II					
Ultrogel AcA 54	2678	158384	59·1	39·3	7·4
Phenyl Sepharose	2219	135362	61·0	33·7	7·6

protein. Purification procedures reported elsewhere for fungal xylanase enzymes have yielded 40–50% of the original activity (e.g. Baker *et al.*, 1977, Yoshioka *et al.*, 1981; Marui *et al.*, 1985; Matsuo & Yasui, 1985; Tan *et al.*, 1985). Labavitch & Greve (1983) reported 9·3% recovery for a xylanase purified from culture filtrates of *T. viride*, but this enzyme was only a minor component of the total xylanase activity separated by chromatography on SP-Sephadex. Similarly, Hashimoto *et al.* (1971) reported a 16·4% recovery of activity for one of two xylanase enzymes separated from *T. viride* culture filtrates. Except for their behaviour on CM-Sepharose chromatography, the xylanases purified from Bioxylanase could not be distinguished by any other property examined. They showed identical isoelectric focusing patterns with a single major protein band (pI 7·3) (Fig. 4). Electrophoresis of either enzyme under denaturing conditions yielded two polypeptide bands of approximately equal intensity with apparent molecular weights of 19900 ± 900 and 16000 ± 900, and a minor band with an apparent molecular weight of 13100 ± 600 (data not shown). This behaviour was unexpected since each enzyme chromatographed as a single sharp peak on gel filtration media (e.g. Fig. 2). This unexpected electrophoretic behaviour could be due to the presence of polypeptide species with quite different patterns of glycosylation or to the removal of smaller peptide fragments during sample preparation for SDS-gel electrophoresis. Of these, the latter would seem more likely because the degree of glycosylation of these proteins is quite low (refer over).

Alkaline pIs are typical of xylanase enzymes purified from *Trichoderma* spp. culture filtrates (Sinner & Dietrichs, 1975; Baker *et al.*, 1977;

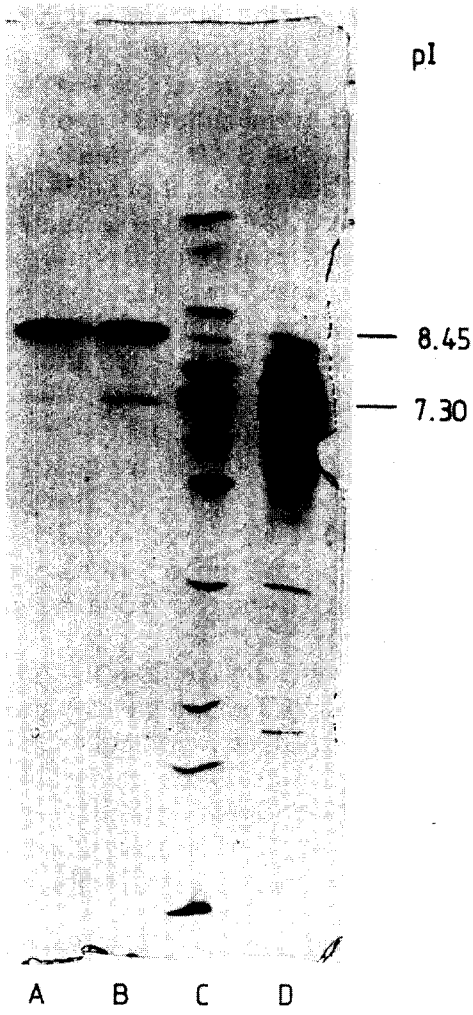

Fig. 4. Isoelectric focusing patterns of xylanase I and II on an LKB PAG plate electrofocusing gel (pH 3·5–9·5). Electrofocusing conditions were according to the manufacturers' instructions. (A) xylanase I; (B) xylanase II; (C) Pharmacia broad pI calibration kit standards (pI 3–10); (D) haemoglobin. All samples were applied at the cathode end.

Labavitch & Greve, 1983; Tan *et al.*, 1985). Interpretation of the molecular weight data in this study for xylanases I and II is more difficult. Molecular weights for xylanase enzymes purified from fungal preparations range from 76 000 for one of the three xylanases produced by *Talaromyces byssochlaonydoides* (Yoshioka *et al.*, 1981) to 7000 for xylanase purified from *Gliocladium virens* cultures (Takahashi & Kutsami, 1979). This wide range may be artefactual, due to the apparent affinity of some xylanases for gel filtration media used for molecular weight determinations (Baker *et al.*, 1977; Marui *et al.*, 1985; Tan *et al.*, 1985). For *T. viride* xylanases, molecular weights of enzymes with activity on

CM-cellulose are 35000 to 44000. Xylanases with no CM-cellulase activity are smaller, with molecular weights of 13000 to 18000 (Toda *et al.*, 1971; Sinner & Dietrichs, 1975; Labavitch & Greve, 1983). This pattern is also shown by xylanases purified from cultures of other *Trichoderma* species (Baker *et al.*, 1977; Tan *et al.*, 1985). Xylanases I and II showed optimal activity on rye flour arabinoxylan at pH 4·5–5·0 and 50°C, and were stable over a pH range of 3·4–7·9 and at temperatures below 55°C (data not shown). These properties are typical of fungal xylanases (Dekker & Richards, 1976). The enzymes were tolerant of acidic assay conditions. Approximately 90% maximum activity was detected at an assay pH of 3·4. No residual activity was detected after a 15 min incubation above 65°C.

The metal ions examined had little effect upon xylanase activity. Cu^{2+}, arsenate and EDTA were slightly stimulatory (15–30% above control activity). Unlike the results obtained with several other fungal xylanases (Kanda *et al.*, 1985; Takahashi & Kutsami, 1979), Hg^{2+} was not inhibitory. The enzymes also contained an unusually small amount of carbohydrate (0·02–0·04%) compared to other fungal xylanases (Toda *et al.*, 1971; Yoshioka *et al.*, 1981).

Purified xylanases I and II had specific activities on rye flour arabinoxylan of 61 U mg^{-1} protein and apparent K_ms of 3·33 mg ml^{-1} on rye flour arabinoxylan and 1·33 mg ml^{-1} on larch xylan. Contaminating activities are shown in Table 2. Most activities were below detectable levels. Unlike purified xylanase from *Cryptococcus albidus* (Biely *et al.*, 1980) no xylosidase activity was detected. Both xylanases readily hydrolyse CM-cellulose 4M6F, but with a lower specific activity than for rye flour arabinoxylan (Fig. 5).

The structure of (1→4)-β-D-xylan differs from that of cellulose only in the presence of the hydroxymethyl group at C-5 on the D-glucosyl residues of cellulose. For the xylanases described here, the presence or absence of this group would appear to have little effect on enzyme–substrate interactions *per se*. However, substitutions on the (1→4)-β-D-glycan main chain by branch substitutions such as L-arabinofuranosyl groups (in arabinoxylan) or carboxymethyl groups (in CM-cellulose) do limit the degree of hydrolysis. This is shown by the resistance of highly branched CM-cellulose 7M to hydrolysis (Fig. 5).

Exhaustive enzymic hydrolysis of rye flour pentosan, in which 70% of the xylosyl residues are substituted with L-arabinosyl groups, yielded mainly high molecular weight products which eluted near the void volume after chromatography on Bio-Gel P-2. Potentially, analysis of reaction products is further complicated by transglycosylation reactions (e.g. Biely *et al.*, 1981). However, hydrolysis of larch wood xylan, with a

TABLE 2
Substrate Specificity for Xylanase I and II

Substrate	*Xylanase I* $U\ ml^{-1}$ *Enzyme*	*Xylanase II* $U\ ml^{-1}$ *Enzyme*
Rye Pentosan	225	210
Starch	<0·0007	<0·0007
Carob Galactomannan	<0·0007	<0·0007
Konjac Glucomannan (native)	<0·0007	<0·0007
Konjac Glucomannan (deacetylated)	<0·0007	<0·0007
Barley β-glucan	<0·0007	<0·0007
Hydrocellulose	<0·0007	<0·0007
Tamarind Amyloid	<0·0007	<0·0007
p nitrophenyl α-D-Glc p	<0·001	<0·001
p nitrophenyl β-D-Glc p	<0·001	<0·001
p nitrophenyl β-D-Xyl p	<0·001	<0·001
p nitrophenyl α-L-Ara f	<0·001	<0·001
p nitrophenyl β-D-Man p	<0·001	<0·001
p nitrophenyl α-D-Gal p	<0·001	<0·001
p nitrophenyl β-D-Gal p	<0·001	<0·001
Hide Powder Azure	<0·001	<0·001

Xylanase activity on all polysaccharides was determined with the appropriate substrate under otherwise standard assay conditions.

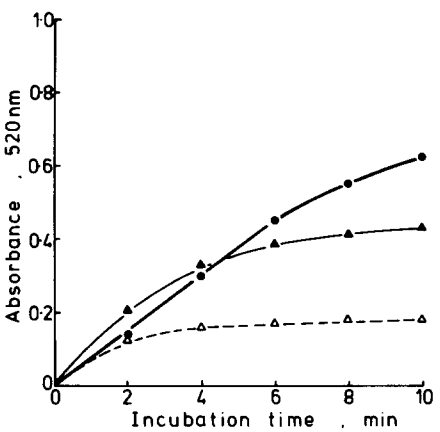

Fig. 5. Time course of reaction of Bioxylanase xylanase I on rye pentosan (●), CM-cellulose 4M6F (▲), and CM-cellulose 7LF (△). Substrate concentration was 5 mg ml^{-1} in 0·1 M sodium acetate buffer (pH 4·5). Incubations were performed at 30°C in the presence of 38 mU (pentosan), 76 mU (CM-cellulose 4M6F) or 760 mU (CM-cellulose 7LF) of xylanase I.

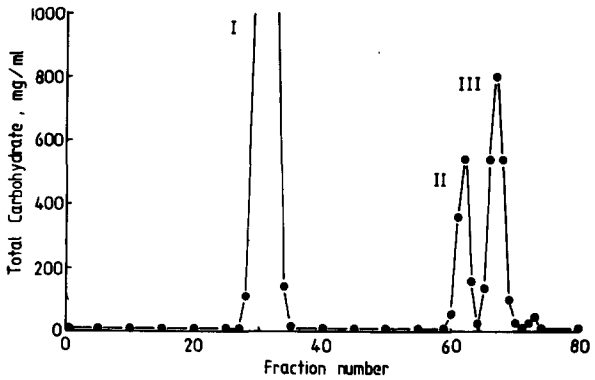

Fig. 6. Chromatography of the oligosaccharides produced by hydrolysis of larch wood xylan by 380 U xylanase I for 24 h at 30°C on a column of Bio-Gel P-2 (<400 mesh); (2·5 × 84 cm). Elution was with H_2O at 60°C. Fractions of 4 ml were collected and analysed for total carbohydrates and reducing sugars. Peaks II and III represent xylotriose and xylobiose respectively. Peak I corresponds to higher molecular-weight xylan-type material.

low degree of substitution, produced low molecular weight material (peaks II and III in Fig. 6). These peaks contained only xylosyl residues when analysed as alditol acetates by gas chromatography. Quantitative assays for total carbohydrate (Dubois *et al.*, 1956) and reducing sugars (Somogyi, 1952) indicated that the oligomers in peaks II and III were xylotriose and xylobiose respectively. These products are typical of those released after xylan hydrolysis by fungal xylanases (e.g. Toda *et al.*, 1971; Dekker & Richards, 1976; Sinner & Dietrichs, 1976; Takahashi & Kutsami, 1979; Marui *et al.*, 1985; Matsuo & Yasui, 1985).

The xylanase enzymes purified from Bioxylanase have physical properties similar to those of xylanases from other *T. viride* preparations (Hashimoto *et al.*, 1971; Toda *et al.*, 1971; Sinner & Dietrichs, 1975; Labavitch & Greve, 1983). However, unlike the enzymes reported previously, the physical and kinetic properties of the two xylanases characterised in this paper were identical, and each exhibited coincident CM-cellulase activity. The high yields of essentially homogeneous *T. viride* xylanases readily purified from commercially available Bioxylanase make this an ideal source of the enzymes for more detailed studies of the structure and function of substituted and non-substituted xylans.

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