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Biochemical characteristics of two endo-β-1,4-xylanases produced by *Penicillium capsulatum*

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SUMMARY

Two endo-β-1,4-xylan xylanohydrolases (EC 3.2.1.8), XynA and XynB, from solid-state cultures of *Penicillium capsulatum*, were purified to apparent homogeneity as judged by electrophoresis and isoelectric focusing. Each is a single subunit glycoprotein. XynA containing 97 mol carbohydrate·mol^{-1} protein, while XynB contains 63 mol·mol^{-1}. M_r and p*I* values are 28500, 5.0–5.2 (XynA) and 29500, 5.0–5.2 (XynB), respectively. Both enzymes are most active at pH 4 and 47–48 °C, and have half-lives of 32 min (XynA) and 13 min (XynB) at pH 4, 60 °C. Each form catalyzed the hydrolysis of a variety of xylans, albeit with different degrees of efficiency. In addition, XynB catalyzed extensive degradation of barley β-glucan, CM-cellulose and, to a lesser extent, lichenan, but kinetic parameters indicate that it is primarily a xylanase. The products of hydrolysis of various xylans and xylopentaose differed for each enzyme and ranged from xylose to xyloheptaose depending on the substrate used. Each enzyme is endo-acting and has transferase as well as direct hydrolase activity. Inactivation by *N*-bromosuccinimide indicated the possible involvement of tryptophan in binding and/or catalysis.

INTRODUCTION

Penicillium capsulatum yields substantial amounts of cellulose-, hemicellulose- and pectin-degrading enzymes when grown on agricultural or forest wastes and residues [10,36]. Moreover, unsupplemented extracts of such cultures effect extensive saccharification of beet pulp [11,13] and successful retting of flax straw [19]. An endopolygalacturonase [18], two endo-β-1,4-glucanases (CM-cellulases) [8] and an unusual carbohydrase complex [9] isolated from such extracts have been characterized and the results of a preliminary investigation of xylan degradation have been reported [17].

The ability of an individual microorganism to provide culture filtrates with all of the activities required for practical applications obviates the inconvenience and expense of supplementation. It is also reasonable to expect that enzymes from *Penicillium* spp. may be approved for use in the food industry. Because of the above, and being mindful of the increasing awareness of the biotechnological potential of xylanases in pulp and paper manufacture [16,30] and in applications in the food industry [25], we have begun investigation of individual components of the xylan-degrading enzyme system of this organism. In this paper we have concentrated on the more readily purified endoxylanases from *P. capsulatum* and report on the characteristics of two such enzymes.

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MATERIALS AND METHODS

Materials

Wheat bran was obtained locally. Beet pulp (average size 0.2 × 0.8 cm) was obtained from Irish Sugar Co., Tuam, Co., Galway, Ireland. Corn steep liquor and barley β-glucan were from Quest-Biocon, Carrigaline, Co., Cork, Ireland; Sephacryl S-100, Sepharose 6B, electrophoretic and isoelectric focusing standard proteins were from Pharmacia LKB (Uppsala, Sweden). DE-52 anion-exchange gel was from Whatman BioSystems Ltd. (Maidstone, Kent, UK); Bio-Gel P-60 was from Bio-Rad Laboratories (Richmond, CA, USA); acrylamide, ammonium persulfate, *p*-hydroxymercuribenzoate (pHMB), CM-cellulose (DS is 0.65–0.85; DP is 200; viscosity of 2% solution at 25 °C is 10–20 cps), Coomassie brilliant blue R250, L-cysteine, diethylpyrocarbonate (DEPC), dithiobis(2-nitrobenzoic acid), dithiothreitol (DTT), laminarin (from *Laminaria digitata*), lichenan (from *Usnea barbata*), 2-mercaptoethanol (MeSH), *N*-bromosuccinimide (NBS),

N-ethyl-5-phenylisoxazolium-3′-sulphonate (Woodward's reagent K), *p*-nitrophenyl-α-L-arabinofuranoside, oat spelts xylan, polygalacturonic acid, phenylglyoxal, polygalacturonic acid, tetramethylenediamine (TEMED), trizma base and L-tryptophan were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Unsubstituted beechwood xylan (DP 30) from the viscose process was obtained from Lenzing AG (Lenzing, Austria).

Arabinoxylan was extracted from delignified wheat straw xylan using NaOH, in the course of which all ester linkages were cleaved [31]. The xylose/arabinose/4-*O*-methylglucuronic acid ratio was 26:2.1:1. Oat spelts and wheat straw arabinoxylans, in 1-g aliquots, were dissolved in 20 ml of 1.0 M NaOH. Twenty ml of 1.0 M HCl were then added and the volumes were brought to 100 ml with 100 mM sodium acetate buffer (pH 5.0). The mixtures were stirred for 1 h at room temperature and then centrifuged for 20 min in a bench top instrument. The supernatants were dialyzed against several changes of distilled water, freeze-dried and used for preparation of stock soluble arabinoxylans. The pellets were washed several times with distilled water and freeze-dried for later use as insoluble arabinoxylans.

A mixed (1,3; 1,4)-linkage xylan was extracted from *Rhodymenia palmata* using HCl as outlined previously [3]. Feruloxylxylan was obtained by steaming extraction of wheat straw [36]. It contained 1.8% acetic acid, 0.4% ferulic acid and 0.1% coumaric acid and its xylan backbone was partially fragmented. Acetyl-4-*O*-methylglucuronoxylan (acetyl content 13%) was obtained by dimethylsulfoxide (DMSO) extraction of beechwood holocellulose [32]. Xylooligosaccharides were prepared as described previously [31]. Type III specific capsular polysaccharide of *Streptococcus pneumoniae* was isolated [20] from appropriate culture fluid kindly provided by Dr. Martin Cormican (Dept. of Bacteriology, University College Hospital, Galway, Ireland). The carboxyl groups of the purified polysaccharide (S III) were reduced via a carbodiimide derivative using sodium borohydride to yield a neutral β-glucan (RS III) with alternating β-1,3 and β-1,4 linkages [2].

Microorganism and cultivation conditions

The organism used was isolated in this laboratory from aerially-contaminated samples of beet pulp and identified as *Penicillium capsulatum* (Raper and Fennell, CMI 291669) by the Commonwealth Mycological Institute, London, UK. We thank the Institute, with whom cultures have been deposited, for identification of the isolate. The procedure for solid-state cultivation was as described previously [10]. The supplemented substrate, adjusted to pH 4.5 with NaOH, had the following composition (w/v): an equal mixture of dried beet pulp and wheat bran, 30% (w/v); distilled water, approx. 67% (v/v); ammonium sulfate, 1.5% (w/v); corn steep liquor, 0.5% (w/v); yeast extract (0.1%, w/v), KH_2PO_4 (0.5%, w/v) and mineral salts (1%, v/v) [28].

Enzyme extraction and analyses

The contents of the solid-state culture flasks were extracted with 10 vol of 25 mM sodium acetate buffer (pH 5), containing 0.01% (v/v) Tween 80, by blending for about 20 s in a homogenizer and then incubating with shaking at room temperature for 2 h [37]. The mixture was then centrifuged for 1 h at $1300 \times g$. The supernatant, hereinafter called the crude extract, was filtered through glass wool and used for purification of the relevant enzymes. For convenience all enzyme assays, except where noted otherwise, were carried out at 50 °C in 0.1 M sodium acetate buffer (pH 5) usually in a total volume of 0.6 ml. Reducing sugars were measured using the dinitrosalicylate method [27]. The hydrolysis of soluble oat spelts xylan (1% w/v) or other polysaccharide (generally 1.0%, w/v) was determined by measuring the release of reducing sugars following incubation of with an aliquot of enzyme for 30 min. Sugars appropriate to the substrate being assayed were used as standards. Arabinofuranosidase activity was determined by monitoring the increase in A_{410}, resulting from the release of *p*-nitrophenol, following 10 min incubation of an aliquot of neat or diluted enzyme with 1 mM *p*-nitrophenyl-α-L-arabinofuranoside as substrate. Reactions were stopped by the addition of 1 ml of 1 M sodium carbonate and *p*-nitrophenol concentration was calculated by reference to a standard curve. Appropriate controls were included in all assays. Activities are expressed as IU ml^{-1}, i.e., μmol reducing sugar or of *p*-nitrophenol, as appropriate, formed min^{-1} ml^{-1} enzyme solution. Experimental data from kinetic studies were fitted to a rectangular hyperbola by computer. The programme used, written in BASIC and kindly provided by Dr. B.A. Orsi (Trinity College, Dublin), is based on the statistical procedures described by Wilkinson [38] and the FORTRAN programme of Cleland [7].

Because of the presence of phenolic and other interfering substances in crude extracts and fractions during the early stages of purification, protein concentration could be measured with accuracy only after precipitation and redissolution. Accordingly, protein in such samples was precipitated with trichloroacetate (final concentration 5%, w/v), the pellet was redissolved in 0.1 M NaOH containing 2% (w/v) Na_2CO_3 and protein concentration was determined [24]. Standard bovine serum albumin was treated in the same way. When appropriate, protein concentration was also estimated by measuring absorbance at 280 nm. The carbohydrate content of purified enzyme preparations was determined by the phenol-sulphuric acid

method [14]. Pentose (pentosan) was estimated using the ferric-orcinol procedure [5].

pH and temperature optima and stability

The optimum temperature for activity was determined by carrying out standard assays at various temperatures between 30 °C and 80 °C in 100 mM sodium acetate buffer (pH 5). Account was taken of the variation in pH as a function of temperature. Activation energy values ($kJ \cdot mol^{-1}$) were calculated from Arrhenius plots of the data obtained in such experiments. Optimum pH values were determined by monitoring each activity at 50 °C at various pH values between 2–8. The following buffers were used: 100 mM glycine-HCl (pH 2.0–2.5); 100 mM sodium acetate (pH 3.0–6.0); 100 mM sodium phosphate (6.5–7.0). All buffers, regardless of pH, were adjusted to the same ionic strength with NaCl. The stability of each of the purified enzymes was determined by assaying, under standard conditions, the activity remaining after preincubation for various time periods at 60 °C, pH 4.

Electrophoresis and isoelectric focusing

Purified preparations of each enzyme were examined for homogeneity by electrophoresis under denaturing (SDS-PAGE) and non-denaturing conditions in 15% (w/v) polyacrylamide slab gels [23]. Protein bands were detected by staining with Coomassie brilliant blue R250. Staining for glycoprotein was carried out using the Schiff reagent [26]. Replicate non-denaturing electrophoretic gels, incorporating 0.2% (w/v) oat spelts xylan, were stained for protein as above and for xylanase activity using Congo red [33].

The M_r values of the purified enzymes were calculated by comparing their mobilities with those of standard proteins (M_r range 14400–94000) under denaturing (SDS-PAGE) conditions, as described in Pharmacia instruction booklet 11-B-036-05. p*I* values were determined by isoelectric focusing on LKB ampholine PAG plates covering the pH range, 3.5–9.5, using the ampholytes (pH range 3 to 10) supplied by Pharmacia and the procedures recommended in the Pharmacia practical information booklet 1804. Standard pH marker proteins (Pharmacia), covering the range 3.5–9.3, were run in conjunction with the unknowns. Gels were stained for protein and for activity as described above.

Fractionation of the products of xylanase action

The products of hydrolysis of various xylans and of xylopentaose were fractionated, identified and quantitated using high-performance anion-exchange chromatography (Dionex) system as described earlier [32].

RESULTS AND DISCUSSION

Purification of xylanases

One litre of crude extract of was concentrated approx. 10-fold by ultrafiltration at a pressure of < 10 psi using an Amicon DC2 system (hollow fibre HIP 10-43 dialyzer) equipped with a 10 kDa (cut-off point) membrane. The permeate (ultrafiltrate) and retentate were assayed for activity as a matter of course. The only activity found in the filtrate was xylanase (see later), while, as expected, a range of carbohydrase activities was found in the retentate.

The retentate was fractionated by gel filtration on Sepharose 6B (Fig. 1A). Two peaks of activity were obtained. The first of these contained arabinosidase in addition to xylanase activity and has, as yet, been used only for purification of the former enzyme (Filho et al., unpublished results). The xylanase (XynA) in the second peak was purified further. Fractions 71–85 of this peak were pooled, dialyzed at 4 °C overnight against distilled water and freeze-dried.

The freeze-dried sample was fractionated by anion-exchange chromatography on DE-52 cellulose at pH 5.5 (Fig. 1B). One peak of xylanase activity eluted in the pre-gradient, while a second, representing only a small proportion of the total applied activity, eluted at a NaCl concentration of approx. 0.3 M. Fractions (11–24), representing the pre-gradient peak, were pooled, dialyzed at 4 °C overnight against distilled water, freeze-dried and stored at 4 °C for later use.

The permeate (900 ml) from the ultrafiltration step was freeze-dried, dissolved in 50 ml of 100 mM sodium acetate buffer (pH 5.0) and fractionated by gel filtration on Bio-Gel P-60 (Fig. 1C). Fractions 48–66, which contained xylanase activity in apparently homogeneous form (see below) were pooled, dialyzed at 4 °C overnight against distilled water, freeze-dried and stored at 4 °C for later use.

The above purification procedures provided apparently homogenous preparations of two xylanases, one (XynA) from the retentate in a yield of 14% and a 16-fold purification, and the other (XynB) from the permeate in a yield of 12% and a 14-fold purification (Table 1). Since crude extracts contain ancillary enzymes that act synergistically with xylanases to effect hydrolysis of substrate [4,12], the yield and fold-purification values are underestimated.

Homogeneity, M_r and pI values and carbohydrate content

Each enzyme preparation yielded a single band when stained for protein using Coomassie blue following SDS-PAGE on 15% (w/v) slab gels (Fig. 2A and B) or isoelectric focusing (Fig. 2C and D). In each case the single bands staining for protein also stained positively for glycoprotein using the Schiff reagent (not shown). Each prep-

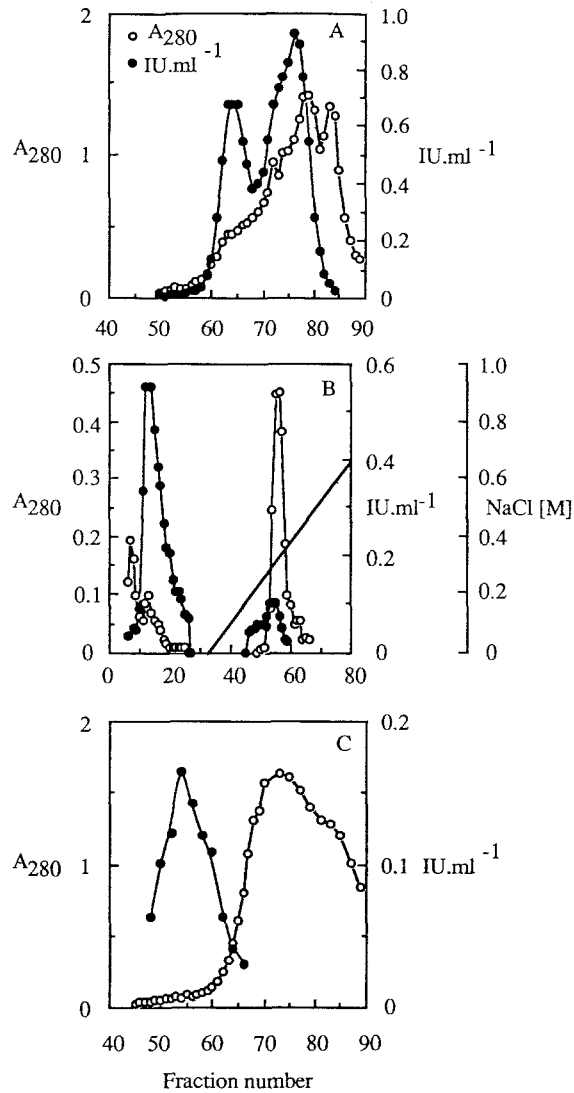

Fig. 1. Purification of two xylanases from extracts of solid-state cultures of *P. capsulatum*. (A) Gel filtration of the retentate on Sepharose 6B. Aliquots (5 ml) of the retentate were fractionated on a column (1.8 × 86 cm) of Sepharose 6B, pre-equilibrated with 100 mM sodium acetate buffer (pH 5.0). Protein was eluted using the same buffer at a flow rate of 14 ml·h^{-1} and fractions of 3 ml were collected. (B) Anion-exchange chromatography on DE-52 at pH 5.5. Fractions 71–85 from the gel filtration step were pooled, dialyzed against distilled water and freeze-dried. The freeze-dried sample was dissolved in 5 ml 100 mM sodium acetate buffer (pH 5.5) and applied to a column (2.5 × 7.2 cm) of DE-52 cellulose previously equilibrated with the same buffer. Protein was eluted by washing with 100 ml of 100 mM sodium acetate buffer (pH 5.5) followed by 200 ml of a linear gradient (0–1.0 M NaCl) in the same buffer, the flow rate and fraction size being 11 ml·h^{-1} and 3 ml, respectively. (C) Gel filtration of the permeate on Bio-Gel P-60. Aliquots (5 ml) of freeze-dried permeate dissolved in 100 mM sodium acetate buffer (pH 5.0) were subjected to gel filtration on a column (1.8 × 82 cm) of Bio-Gel P-60 pre-equilibrated with 100 mM sodium acetate buffer (pH 5.0). Protein was eluted at a flow rate of 26 ml·h^{-1} and fractions of 3.0 ml were collected. In each figure activity is represented by the closed circles, A_{280} by the open circles, while the NaCl gradient in B is represented by the diagonal line.

aration also yielded a single band of protein coincident with a positive stain for activity when subjected to non-denaturing electrophoresis (not shown).

The M_r value of each protein, based on gel filtration on Sephacryl S-100, was calculated to be less than 14000. By contrast, SDS-PAGE indicated the values to be 28500 (XynA) and 29500 (XynB). From the above, one may conclude that each enzyme is a single subunit glycoprotein. The p*I* values for each enzyme were calculated to be 5.0–5.2 on the basis of their relative mobilities during isoelectric focusing.

Carbohydrate contents, perhaps including non-covalently as well as covalently bound material, were determined to be 58.5% (97 mol glucose equivalents·mol^{-1} protein) and 33.4% (63 mol·mol^{-1}), respectively, for XynA and XynB.

pH and temperature optima

Each enzyme was most active at pH 4.0 and 47–48 °C. Energy of activation values, calculated from temperature data, were 15.4 kJ·mol^{-1} and 28.1 kJ·mol^{-1} for XynA and XynB, respectively. The half-life values, calculated from semi-logarithmic plots of residual activity vs. time at 60 °C, pH 4.0, were determined to be 31 min (XynA) and 13 min (XynB).

Substrate specificity and kinetic parameters

Neither of the purified enzymes was active against polygalacturonate or *p*-nitrophenyl-α-L-arabinofuranoside (Table 2). Each enzyme catalyzed the hydrolysis of β-1,4-linked soluble and insoluble arabinoxylans from oat spelts and wheat straw, an unsubstituted xylan with DP of 30, feruloylated xylan, and the mixed (β-1,3;1,4) linkage xylan from dulse (colloq. dilisk; *R. palmata*), although specific activities varied from substrate to substrate and from enzyme to enzyme (Table 2; see also Table 4).

XynA also exhibited low activity against barley β-glucan (a mixed β-1,3;1,4-linked material). However, it was completely inactive against filter paper or Solka floc (β-1,4-linked), CM-cellulose (β-1,4-linked), lichenan (mixed β-1,3;1,4-linked), pneumococcal RS III (alternating β-1,3 and β-1,4 linkages) or laminarin (β-1,3-linked). Taken together, these results suggest that XynA cleaves only β-1,4 linkages, and that it does so only when a stretch of several such linkages occur together providing that the polysaccharide is not substituted by carboxymethyl groups. It remains to be seen whether the observed hydrolysis of arabinoxylan and feruloylated xylan occurs only

TABLE 1

Summary of the purification of XynA and XynB from *Penicillium capsulatum*

Step	Total protein (mg)	Total activity (IU)	Specific activity ($IU \cdot mg^{-1}$)[a]	Purification (-fold)	Yield (%)
Retentate (XynA)[b]					
Ultrafiltration (retentate)	333.3	2009.0	6.0	1.0	100.0
Gel filtration on Sepharose 6B (fractions 71–85)	100.0	980.0	9.8	1.6	48.8
Ion-exchange on DE-52 at pH 5.5 (fractions 11–24)	2.9	276.0	96.6	16.0	13.7
Permeate (XynB)					
Crude extract	360.0	12355.0	34.3	1.0	100.0
Ultrafiltration (permeate)	22.0	3456.9	157.8	4.6	28.0
Gel filtration on BioGel P-60 (fractions 48–66)	3.0	1478.7	492.9	14.4	12.0

[a] With soluble oat spelts xylan substrate. [b] Because concentration of culture filtrates results in an apparetn decrease in measureable activity [39], perhaps by concentrating inhibitors, the yield and fold-purification values of XynA are given relative to activity in the retentate.

at stretches free from substitution or whether main chain linkages close to such substituents may also be cleaved. This would provide information on whether certain substituents may be accommodated at the active site or not.

In addition to soluble and insoluble xylans, XynB also catalyzed extensive cleavage of CM-cellulose, barley β-glucan, laminarin and, to a lesser extent, lichenan, but had almost no activity against RS III (Table 2). The observed action against β-1,3-linked laminarin was surprising even though a xylanase from *Trichoderma reesei* was also found to exhibit high activity against this substrate [22]. The laminarin samples used in this study reacted positively with the ferric-orcinol reagent for pentoses, and may be contaminated with a susceptible pentosan. An alternative possibility, which we do not overlook, is that the enzyme preparation, even though it gives a single band on SDS-PAGE and isoelectric focusing, is contaminated by laminarinase activity. However, further work with new samples of this substrate will be required to decide on this. From the above we conclude that the action of XynB, like that of XynA, is directed towards β-1,4-linkages and that a number of adjacent β-1,4-linkages are required for activity. However, in this case, carboxymethyl substituents (e.g., CM-cellulose) may be accomodated in the active site.

Kinetic parameters

The kinetic parameters of both enzymes acting on several substrates are listed in Table 3. It may be noted that, while the k_{cat} values (turnover numbers) for XynA are considerably greater than those for XynB, the $k_{cat} \cdot K_m^{-1}$ (specificity constants) values for both enzymes are comparable with xylans as substrates. In the case of XynB (the less specific of the two enzymes), comparison of k_{cat} and $k_{cat} \cdot K_m^{-1}$ values obtained with xylans and with those using CM-cellulose as substrate clearly show that this enzyme also is primarily a xylanase.

Products of hydrolysis of xylans and xylopentaose

So as to facilitate detection, identification and quantitation of intermediate products of xylan hydrolysis, the experimental conditions chosen were such that extensive hydrolysis would not take place (Table 4). Neither enzyme effected the release of free arabinose from any of the appropriate substrates used. The only low molecular weight unsubstituted products detected following reaction of XynA with insoluble wheat straw arabinoxylan were xylobiose and xylotriose, the dimer accounting for 91.3% of the total on a weight basis. With all other substrates, XynA released products ranging from xylose (from acetylxylan and feruloxylan) or xylobiose (from unsubstituted xylan DP 30 and xylopentaose) to xyloheptaose, the relative proportions of each oligomer depending on the substrate (Table 4). Substituted products were also produced but, because of the lack of appropriate standards, have not been quantitated. The above observations are consistent with endo-action. Moreover, the fact that hexamers and heptamers were detected following incubation with xylopentaose indicates that this enzyme catalyzes transfer reactions as well as direct hydrolysis. Comparison of the extents of hydrolysis of the various polymeric substrates by XynA would suggest that some substitutions (e.g., acetyl) are better tolerated than others. The lower degree of hydrolysis of arabinoxylan, feruloylxylan and unsubstituted xylan may reflect steric hindrance by the substituents or poor solubility of the substrate, or both. The relatively low degree of hydrolysis of xylopentaose may be due in part to the operation of transferase activity, product inhi-

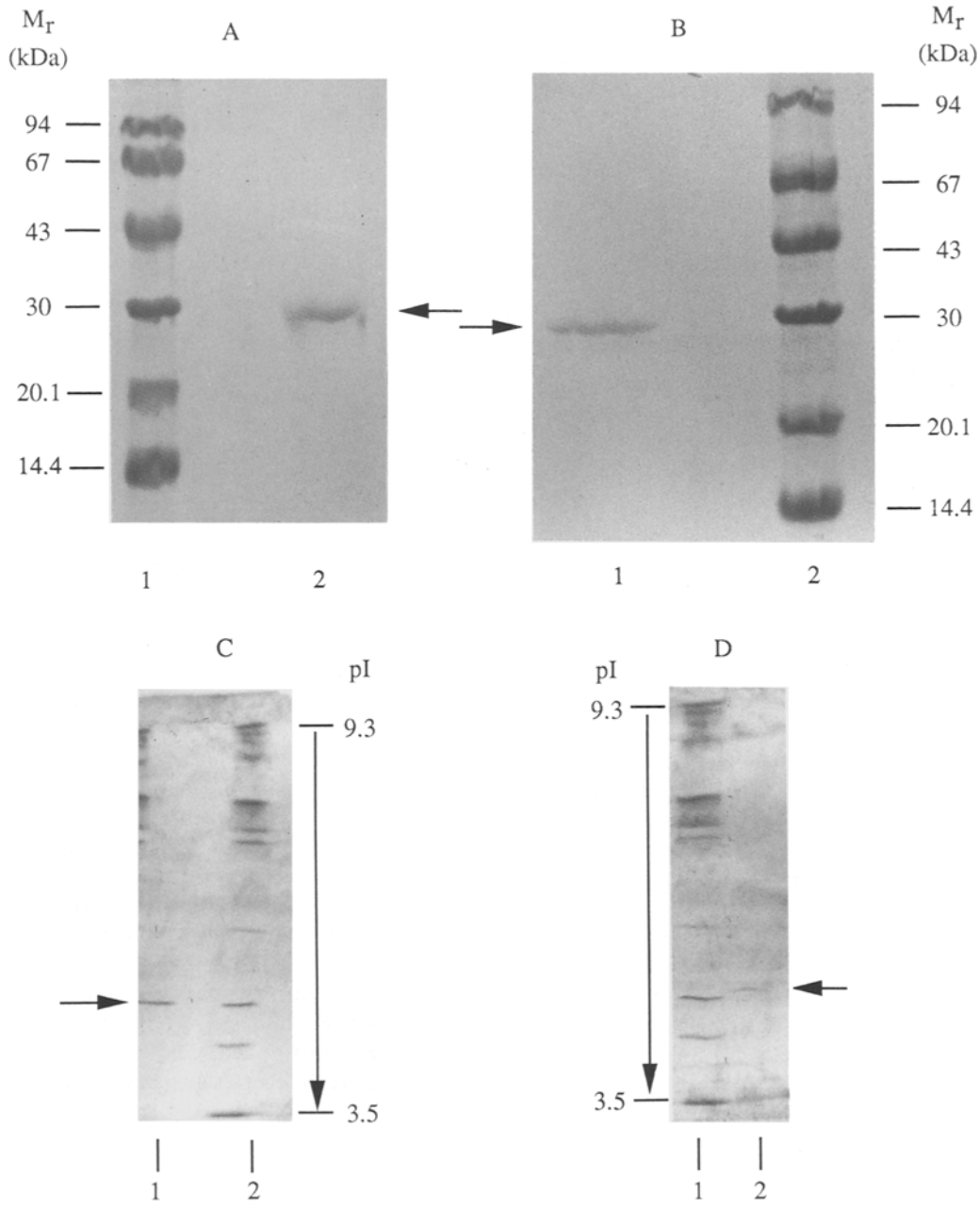

Fig. 2. Electrophoresis and isoelectric focusing of XynA and XynB from *P. capsulatum*. (A) SDS–PAGE of XynA on 15% slab gel. Lane 1, standard marker proteins; lane 2, specific enzyme. (B) SDS-PAGE of XynB on 15% slab gel. Lane 1, XynB; lane 2, standard marker proteins. (C) Isoelectric focusing of XynA (lane 1) and p*I* standards (lane 2). (D) Isoelectric focusing of pI standards (lane 1) and XynB (lane 2). On isoelectric focusing gels each protein migrated at approximately the same rate as the standard p*I* 5.2 marker. However, least squares computer fitting of the migration of all standards indicated that both had p*I* values of 5.0.

bition or to the fact that substrates with DP > 5 are preferred.

The relative extents to which the polymeric substrates were degraded by XynB were more uniform (Table 4). This may indicate that XynB is better equipped to accomodate substituents at or near the active site than is XynA. Consistent with this possibility is the fact that only XynB was active against CM-cellulose (Table 3). In all cases, the unsubstituted low molecular weight products produced ranged from xylose to xyloheptaose. These observations are also consistent endo-action. XynB also catalyzes transfer reactions as well as direct hydrolysis.

TABLE 2

Substrate specificities of two xylanases from *Penicillium capsulatum*

Substrate	Main chain linkage	XynA	XynB
		relative activity[a]	
Oat spelts xylan (soluble)	β-1,4	100.0	100.0
Oat spelts xylan (insoluble)	β-1,4	37.3	37.6
Wheat straw xylan (soluble)	β-1,4	67.9	95.6
Wheat straw xylan (insoluble)	β-1,4	95.7	180.3
Rhodymenia palmata xylan	β-1,4 (82%); β-1,3 (18%)	124.4	123.0
Cellulose (filter paper)	β-1,4	0	0
CM-cellulose	β-1,4	0	23.3
Barley β-glucan	β-1,4 (75%); β-1,3 (25%)	4.9	89.4
Pneumococcal RS III	alternating β-1,4 and β-1,3	0	1.0
Laminarin	β-1,3	0	18.7
Lichenan	β-1,4 (65%); β-1,3 (35%)	0	5.0
Polygalacturonate	α-1,4	0	0

[a] The samples of XynA and XynB used had 10.4 and 5.3 IU·ml^{-1}, respectively, as measured with soluble oat spelts xylan as substrate. These values were arbitrarily assigned as representing 100% activity in each case.

Effects of amino acid modifying reagents

The fact that each enzyme was activated by thiol containing reagents (cysteine, mercaptoethanol (MeSH) and dithiothreitol (DTT) (Table 5) may suggest that reduction of a disulfide(s), perhaps oxidized during extraction and purification, restores the native enzyme conformation generally or of the active site region in particular. The failure of iodoacetamide or dithionitrobenzoic acid (DTNB) to effect inactivation, and the fact that 10 mM *p*-hydroxymercuribenzoate (pHMB) effected relatively little inactivation, may indicate that cysteine is either not present at the active site or, if present, is inaccessible to the reagent. Lack of inactivation by diethylpyrocarbonate (DEPC) may also rule out the involvement of histidine in binding or catalysis.

By contrast, inactivation by *N*-bromosuccinimide (NBS), especially of XynA, initially suggests the involvement of tryptophan in one or other, or both, of these two processes, or that it is involved in maintaining the integrity of the active site. The fact that substrate protects XynB from inactivation by NBS is consistent with tryptophan being involved in binding or catalysis by this enzyme. By the same line of reasoning, the failure of substrate to protect XynA against inactivation may rule out a direct involvement of tryptophan in this enzyme. Cysteine, MeSH or DTT protected both enzymes against inactivation by NBS but did not reverse such inactivation once established. One may note that side reactions of NBS include oxidation of cysteine [34]. However, the failure of the thiol-containing agents above to reverse in-

TABLE 3

Kinetic properties of XynA and XynB from *Penicillium capsulatum*

	XynA		XynB		
	(substrate)		(substrate)		
	OSX[a]	WSX[a]	OSX[a]	WSX[a]	CMC
K_m (mg·ml^{-1})	46.0	33.7	7.0	9.8	7.1
(mM)[b]	(348.0)	(255.0)	(52.7)	(74.2)	(44.0)
V_{max} (IU·ml^{-1})	143.3	75.5	13.3	10.8	1.6
k_{cat} (s^{-1})	619.0	326.0	93.0	76.0	11.0
$k_{cat}\cdot K_m^{-1}$ (s^{-1}·mM^{-1})	1.8	1.3	1.8	1.0	0.2

[a] OSX, soluble oat spelts xylan; WSX, soluble wheat straw xylan. [b] Calculations based on anhydroxylose or anhydroglucose content as appropriate.

TABLE 4

Products of hydrolysis of various substrates by XynA and XynB

Substrate	Hydrolysis (%)[b]	X_1	X_2	X_3	X_4	X_5	X_6	X_7
		as μg% of total unsubstituted xylooligomers (DP 1–7) produced						
XynA								
Acetylxylan	21.0	1.3	8.3	17.1	23.0	16.6	19.4	14.4
Arabinoxylan (insol.)	1.9	0	91.3	8.7	0	0	0	0
Feruloylxylan	3.9	21.6	2.3	10.9	13.5	15.5	16.7	19.5
Unsubstituted xylan	5.3	0	1.8	15.9	18.5	21.4	20.5	22.0
Xylopentaose (1:20)[a]	7.0	0	1.0	1.8	4.3	92.9	0	0
Xylopentaose (1:10)[a]	19.1	0	2.9	4.8	5.4	80.9	1.5	4.5
XynB								
Acetylxylan	18.8	0.5	6.5	16.8	22.4	17.2	20.2	16.5
Arabinoxylan (insol.)	9.4	1.0	19.2	45.3	19.6	8.7	4.0	2.2
Feruloylxylan	19.0	0.5	8.9	27.1	22.6	18.2	13.1	9.6
Unsubstituted xylan	16.1	1.5	14.9	38.3	19.4	12.6	0	13.2
Xylopentaose (1:20)[a]	61.0	0	12.5	28.4	15.2	28.9	8.8	6.3
Xylopentaose (1:10)[a]	91.0	0.7	18.1	40.2	19.1	12.7	5.8	3.5

Reaction mixtures containing 10 mg of polymeric substrate plus 7 μg of XynA, or 28.5 μg of XynB, per ml of 100 mM sodium acetate buffer (Ph 5) were incubated for 12 h at 50 °C. [a] In the reactions with xylopentaose (5 mg·ml^{-1}) the amounts of enzyme used were 10- or 20-fold less than those used for incubation with polymeric substrates. [b] As measured by the total amount of unsubstituted xylooligomers (DP 1–7) released.

TABLE 5

Effects of various agents on the activities of XynA and XynB

Reagent	Conc. (mM)	XynA	XynB
		relative activity (% control)	
Cysteine	10	150	181
DTT	10	156	212
MeSH	10	138	161
DTNB	2	100	100
pHMB	10	50	73
Iodoacetamide	10	100	100
Iodine	2	90	91
DEPC	20	100	100
Tryptophan	10	144	144
NBS	0.5	1.4	25
NBS + xylan[a]	0.5 + (4 mg·ml^{-1})	7.0	61

Enzyme samples were incubated with the indicated agents (concentration shown) for 1 h at 25 °C in 100 mM sodium acetate buffer (pH 5). Aliquots were then taken for assay of activity under standard conditions. [a] Oat spelts xylan was mixed with enzyme just before addition of the inhibitor and incubation was carried out as above. Appropriate controls were included in all cases. Abbreviations used are defined in the text.

activation by NBS would appear to rule out the occurrence of the side reaction in question in this case. Rather we presume that the protection by the thiols against NBS results from direct reaction between the reducing thiols and the strongly oxidizing NBS.

The finding by Clarke [6] of two functionally distinct tryptophan residues in a cellulase from *Schizophyllum commune* may be pertinent to the above discussion. In this enzyme one tryptophan residue was stated to be directly involved in substrate binding, while a second, also at the active site, is part of the catalytic mechanism. The binding of substrates or competitive inhibitors protected one tryptophan residue against oxidation by NBS but did not prevent inactivation. Moreover, it was concluded that most substrates or competitive inhibitors make contact with only one of the two tryptophans and, while masking it from NBS action, expose the second to attack [6].

We are at a loss to explain the observation that preincubation with tryptophan effected considerable activation of each enzyme above (Table 5). Somewhat tongue in cheek, we suggest the possibility that perturbation of an essential tryptophan during the reaction, or modification of such a residue by some unknown component of the reaction mixtures, or its oxidation during extraction and purification may be reversed or prevented by the added tryptophan.

Comparison with other xylanases

The comparative physicochemistry and biochemistry of bacterial and fungal xylanases has been the subject matter of several recent reviews [12,15]. In the interests of conserving space, one might summarize their properties as follows. Reported M_r values range from 8.5–85.0 kDa (very low values generally have been obtained by gel filtration and should be viewed with caution), while p*I* values range from 4 to 10.3. Indeed, Wong et al. [40] noted that xylanases generally fall into two classes, acidic/high M_r values (>30000) and basic/low M_r values (<30000), although there were exceptions. Clearly the two enzymes reported here are among the exceptions. The finding that proteins with M_r values in excess of the nominal cut-off of Amicon filters can permeate such filters, as was the case with XynB, has been observed before. Indeed, this fact has been exploited in the facile preparation of a cellulase-free endoxylanase from culture filtrates of *Trichoderma harzianum* [35].

Most xylanases have acidic to neutral pH optima, 3.5–7.0, although a few examples of bacterial xylanases with optimal activity at pH values between 7.0 and 9.0, notably those from an alkalophilic thermophilic *Bacillus* sp. [1], have been reported. K_m values, generally measured with oat spelts xylan as substrate range from 0.78 to 10.0 $mg \cdot ml^{-1}$, but it must be said that the range among the fungal enzymes is less pronounced. With arabinoxylans as substrates, hydrolysis products include xylooligomers with DP values ranging from 1–6 as well as arabinose-substituted xylooligomers. Neither XynA nor XynB hydrolyzed *p*-nitrophenyl-α-L-arabinoside nor released free arabinose from arabinoxylan. However, quite a high percentage of xylanases have been reported to liberate free arabinose from the latter substrate, even though they do not exhibit arabinosidase activity against artificial substrates (see Ref. 12 for review). Whether this represents a real activity of such xylanases (mechanistically it is difficult to see how this could be so) or due to chance contamination, with an enzyme such as arabinoxylan arabinofuranohydrolase [21], has not been resolved. In this context it has been suggested that examination of the products of hydrolysis by cloned enzymes may be needed to clarify the situation [12].

Some xylanases catalyze transferase activity as well as direct hydrolysis, while others do not; some act against substrates such as CM-cellulose as well as xylans, while others are restricted in their action to xylans; some are particularly prone to endproduct or excess substrate inhibition; some effect the hydrolysis of insoluble as well as soluble substrates, although most xylanases act only against the soluble materials. Finally, the actions of some xylanases are hindered by substitutions, i.e., they act only at unrestricted sites on the xylan backbone, while others only cleave backbone linkages at or near points of substitution (for a review see Ref. 12). One striking example of this is a glucuronoxylan xylanohydrolase that has an absolute requirement for glucuronic acid on the xylosyl unit adjacent to that comprising the new reducing group [29]. Thus, some xylans are resistant to hydrolysis and, as the authors rightly point out, would be overlooked by most routine assays. Further work will be required to determine the influence of various types of substitution on the activities of the two xylanases reported on here and on the mechanisms whereby they effect hydrolysis of the substrate.

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