Carbohydrate Research, 128 (1984) 141–155
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

PURIFICATION AND PROPERTIES OF AN ENDO-(1→4)-β-D-XYLANASE FROM *Irpex lacteus (Polyporus tulipiferae)*

CHRISTINE HOEBLER AND JEAN-MARC BRILLOUET*

Institut National de la Recherche Agronomique, Centre de Recherches Agro-Alimentaires, Laboratoire de Biochimie et Technologie des Glucides, Rue de la Géraudière, 44072 Nantes Cédex (France)

(Received July 28th, 1983; accepted for publication, November 15th, 1983)

ABSTRACT

A xylanase from Driselase (a commercial enzyme preparation), obtained from the basidiomycetes *Irpex lacteus (Polyporus tulipiferae)*, was purified ~32-fold by desalting on Sephadex G-25, ion-exchange chromatography on DEAE-Sepharose CL-6B and CM-Sepharose CL-6B, hydrophobic-interaction chromatography on Phenyl-Sepharose CL-4B, gel filtration on Ultrogel AcA54, and affinity chromatography on Concanavalin A-Ultrogel. The enzyme is a glycoprotein that contains 23% of sugars, mainly as glucose. Its molecular weight is 38,000 and its pI 7.6–8.0. The enzyme exhibited maximal activity at pH 4.6–5.2 and at 60°, and was completely inactivated within 30 min at 70°. The K_m values for larch 4-*O*-methylglucuronoxylan were 2.8 (suspension in water) and 1 mg/mL (solution in 20% methyl sulfoxide). The xylanase degraded larchwood xylan to xylose, xylobiose, and xylotriose, as neutral end-products.

INTRODUCTION

Xylans are the major hemicellulosic polysaccharides occurring in such lignocellulosic waste-materials[1] as straws from the Gramineae, bagasse, and husks. They contain a backbone of (1→4)-linked β-D-xylosyl residues to which are attached[2,3] single 4-*O*-methyl-D-glucosyluronic acid groups at O-2 and/or α-L-arabinofuranosyl groups at O-3, or extended side-chains carrying arabinosyl and xylosyl residues[4].

Xylanases (EC 3.2.1.8.) are endo-enzymes, which can cleave the (1→4)-β-D-xylopyranosyl linkages of xylans, and are found mainly in fungi, but also occur in bacteria, insects, and plants[5]. Xylanases associated with cellulases are the major components responsible for the biodegradation of lignocellulosic materials. The use of homogeneous xylanases as highly specific tools for elucidating the structure of alkali-extracted xylans[6], as well as the organisation of intact cell-walls of plants[7], is very promising.

*To whom correspondence should be addressed.

0008-6215/84/$ 03.00

Nisizawa *et al.*[8–10] and Kanda *et al.*[11–16] have studied in detail the cellulases from the basidiomycetes *Irpex lacteus (Polyporus tulipiferae)*. We now describe the purification and properties of an endo-(1→4)-β-D-xylanase from this fungus.

RESULTS AND DISCUSSION

Purification (See Table I). — Ion-exchange chromatography of a xylanase from *Irpex lacteus (Polyporus tulipiferae)* on DEAE-Sepharose CL-6B gave a 5-fold purification and removed most of the coloured material. Of the original xylanolytic activity, ~67% remained unbound, and ~80% of the original protein material was eliminated.

Further ion-exchange chromatography on CM-Sepharose CL-6B at pH 4.4 gave a major, broad xylanase peak that was eluted by ~0.15M sodium chloride (Fig. 1). It contained ~39% of the original activity, and ~80% of the injected proteins were removed (purification 14-fold). Successive dialyses against mM phosphate buffer (12 h, pH 7.0) and 20mM acetate buffer (2.5 h, pH 4.4) were necessary for reproducibility of the subsequent chromatography on CM-Sepharose. The du-

TABLE I

PURIFICATION OF XYLANASE FROM COMMERCIAL "DRISELASE"

Step	*Volume (mL)*	*Activity (nkat. mL^{-1})*	*Protein (mg. mL^{-1})*	*Specific activity (nkat. mg^{-1})*	*Total activity (nkat)*	*Yield (%)*	*Purification (fold)*
Crude enzyme	23	1404	57.8	24.3	32300	100	—
Desalting on Sephadex G-25M	66	478	14.1	34.0	31570	97.7	1.4
Ion-exchange chromatography on DEAE-Sepharose CL-6B	158	138	1.13	122	21750	67.3	5
Ion-exchange chromatography on CM-Sepharose CL-6B	196	63.7	0.19	335	12490	38.7	14
Hydrophobic-interaction chromatography on Phenyl-Sepharose CL-4B	100	78.3	0.12	652	7834	24.2	26
Gel filtration on Ultrogel AcA54	26	227	0.31	732	5905	18.3	30
Affinity chromatography on Concanavalin A–Ultrogel AcA22	63	50.6	0.065	778	3188	9.9	32

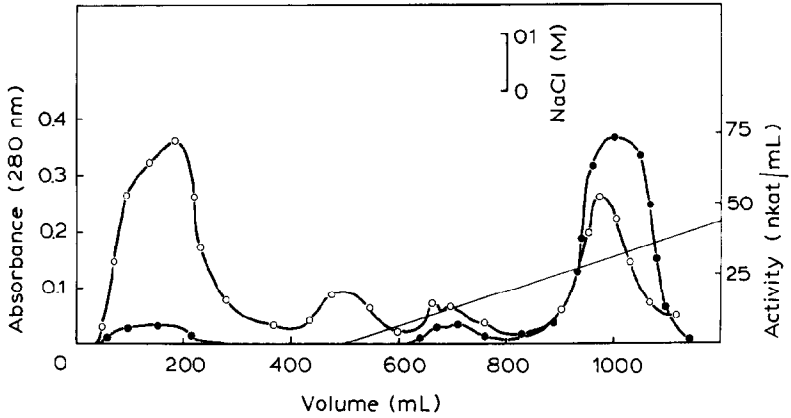


Fig. 1. Ion-exchange chromatography of the *Irpex lacteus* xylanase on CM-Sepharose CL-6B (see Experimental). Fractions (5 mL) were assayed for protein at 280 nm (—○—) and xylanase activity (—●—).

ration of the second dialysis step was critical, because the xylanase was unstable under these conditions.

Attempts to purify the xylanase by chromatofocusing on PBE 118 (Pharmacia) were unsuccessful, because most of the activity did not bind to the exchanger even at a pH as high as 11.0. Thus, the enzyme had a low density of ionisable groups over a wide pH-range; on hydrophobic-interaction chromatography, it was bound to Phenyl-Sepharose CL-4B, ~62% of the injected activity being eluted as a sharp peak (Fig. 2) at ~25% ethylene glycol (~37% of injected protein). An inactive fraction of low molecular weight was eliminated by gel filtration on Ultrogel AcA54 (Fig. 3).

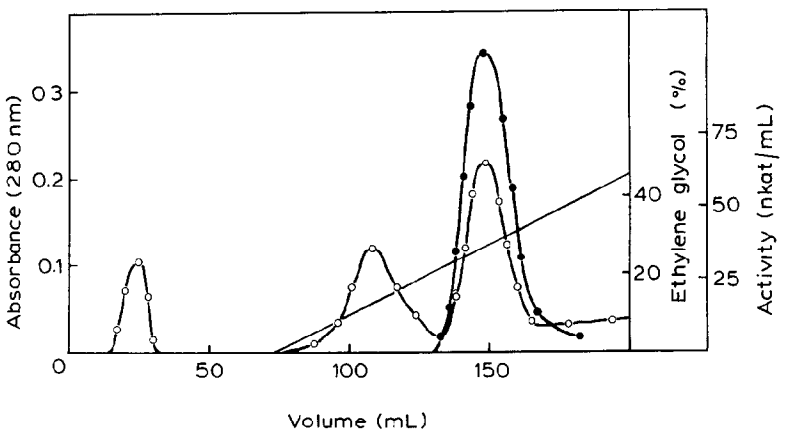


Fig. 2. Hydrophobic-interaction chromatography of the xylanase on Phenyl-Sepharose CL-4B (see Experimental). Fractions (2 mL) were assayed for protein at 280 nm (—○—) and xylanase activity (—●—).

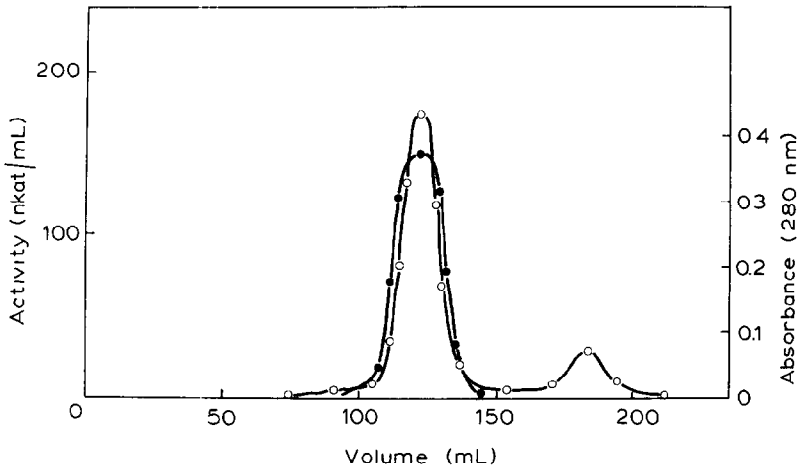


Fig. 3. Gel filtration of the xylanase on Ultrogel AcA54 (see Experimental). Fractions (1.2 mL) were assayed for protein at 280 nm (—○—) and xylanase activity (—●—).

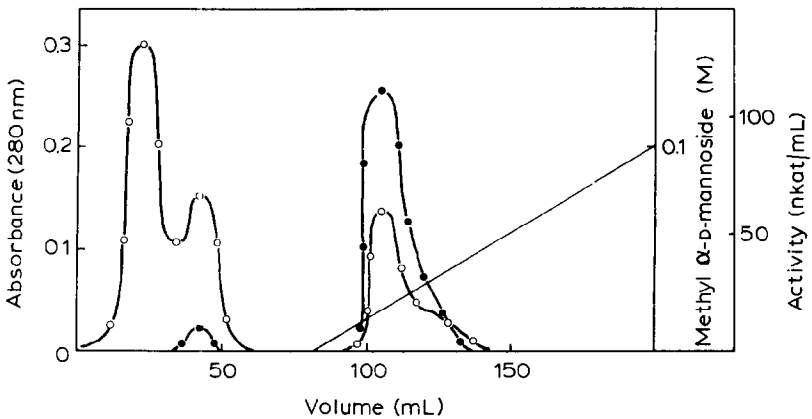


Fig. 4. Affinity chromatography of the xylanase on Concanavalin A–Ultrogel AcA22 (see Experimental). Fractions (1.5 mL) were assayed for protein at 280 nm (—○—) and xylanase activity (—●—).

Final purification involved lectin-affinity chromatography on Concanavalin A–Ultrogel AcA22 (Fig. 4). The xylanase was firmly bound and was not eluted with M sodium chloride. Methyl α-D-mannopyranoside released the xylanase in ~50% yield for both injected protein and activity.

The purified xylanase had ~10% of the original activity (corresponding to a 32-fold purification), had a specific activity of 778 nkat.mg^{-1}, and gave a single, diffused protein band on SDS-polyacrylamide gel electrophoresis and electrofocusing; it had no activity against various oligomeric and polymeric substrates, but showed a very low and variable activity (<0.7%) against *o*-nitrophenyl β-D-xylopyranoside.

Properties. — Some of the properties of the xylanase are reported in Table II. The molecular weight, as determined by SDS-polyacrylamide gel electrophoresis and by gel filtration on Ultrogel AcA54 (Fig. 3), was 38,000. The isoelectric point was difficult to determine, because of the low density of ionisable groups over a wide pH-range, and the enzyme did not focus even after long runs; only a pI range of 7.6–8.0 could be proposed. As compared to other fungal xylanases[17–20], the *Irpex lacteus* xylanase was richest in hydrophobic and acidic amino acids (Table III); it was devoid of cysteine, reflecting the lack of inhibition by *N*-ethylmaleimide (see below). The xylanase contained 23% of sugars. G.l.c. of the derived alditol acetates revealed glucose and mannose in the ratio 13:1. The preponderance of glucose over mannose explains the easy release of the enzyme from Concanavalin A-Ultrogel by methyl α-D-mannopyranoside, because glucose has a far lower affinity for Con A than that of α-D-mannose[21].

TABLE II

PROPERTIES OF XYLANASE

Property	*Xylanase*
Molecular weight[a]	38,000
pI	7.6–8.0
Carbohydrate[b] (%)	23 (Glc/Man = 13:1)
pH optimum	4.6–5.2
θ optimum (°)	60
Larch 4-O-methylglucuronoxylan	
V_{max} (40°, pH 4.2) (nkat.mg^{-1})	824
K_m (40°, pH 4.2) (mg.mL^{-1})	2.8
V_{max} (40°, 20% Me_2SO, pH 4.2)[c] (nkat.mg^{-1})	934
K_m (40°, 20% Me_2SO, pH 4.2) (mg.mL^{-1})	1.0

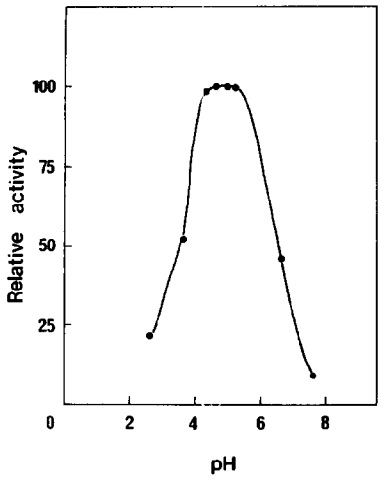
[a]Determined by SDS-polyacrylamide gel electrophoresis. [b]Determined by the phenol–sulfuric acid method. [c]Larch xylan was dissolved in pure Me_2SO and then diluted to 20% in the standard assay.

TABLE III

AMINO ACID PROFILE OF *Irpex lacteus* XYLANASE

Amino acid[a]	*Mole (%)*	*Amino acid*[a]	*Mole (%)*
Aspartic acid	10.8	Isoleucine	2.7
Threonine	5.9	Leucine	7.6
Serine	13.5	Tyrosine	1.3
Glutamic acid	12.3	Phenylalanine	4.1
Proline	4.0	Lysine	4.0
Glycine	13.0	Histidine	2.5
Alanine	11.2	Arginine	1.2
Valine	5.9		

[a]Tryptophan was not determined.



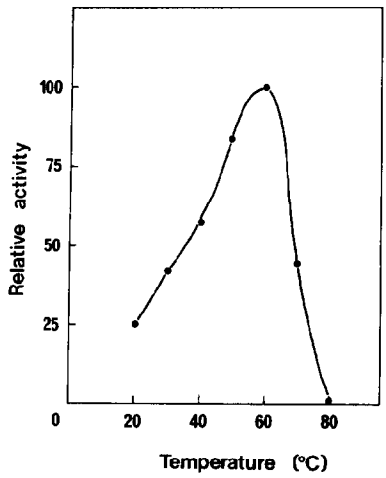
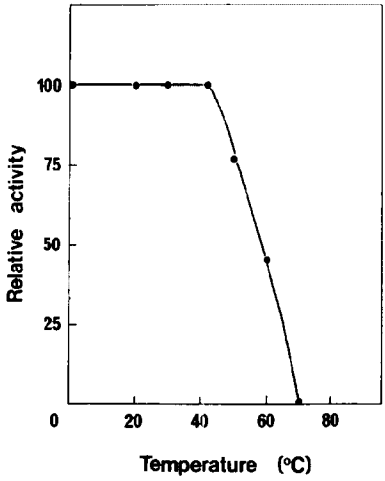


Fig. 5. Effect of pH on enzyme activity (see Experimental).

Fig. 6. Effect of temperature on enzyme activity (see Experimental).



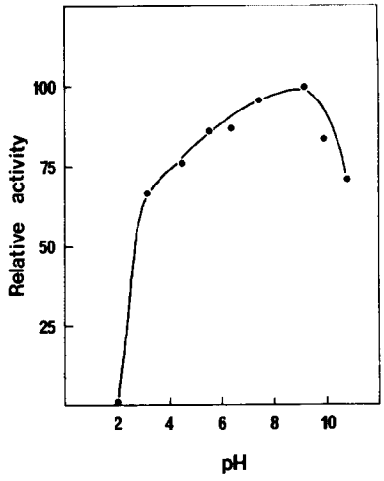


Fig. 7. Effect of temperature on enzyme stability (see Experimental).

Fig. 8. Effect of pH on enzyme stability (see Experimental).

The enzyme exhibited maximum activity in the pH range 4.6–5.2 (Fig. 5) and at 60° in 20-min assays (Fig. 6), was inactivated after 30 min at 70° (Fig. 7), was stable in the pH range 6.5–9.0 (the stability decreasing gradually from pH 6.0 to 3.5), and was rapidly inactivated at pH <3.0 and >10.5 (Fig. 8).

Of the metal ions tested (Table IV), only Ca^{2+} significantly increased the activity (~30%); the activation of xylanases by Ca^{2+} has been reported[22,23]. The enzyme was inhibited by mM Hg^{2+} and Ag^{+}. Inhibition by Hg^{2+} in the absence of cysteine may indicate the involvement of tryptophan units in the maintenance of enzyme activity[23]. There were difficulties in measuring the inhibition caused by Fe^{2+}, because of the formation of insoluble salts with 4-*O*-methylglucuronate from the larch xylan. Tryptophan, which was not determined in this study, must occur in the xylanase because 2-hydroxy-5-nitrobenzyl bromide (Table V), a specific agent for tryptophan, totally inhibited the xylanase activity. As shown by partial inhibition of the enzyme by *N*-acetylimidazole, tyrosine units are probably involved in the xylanase catalysis. EDTA did not significantly inhibit the enzyme. The xylanase from *Irpex lacteus* reacts to these various inhibitors in a manner very similar to that of the *Trametes hirsuta* xylanase.

TABLE IV

EFFECT OF METAL IONS ON XYLANASE

Ion	*Concentration tested (mM)*	*Relative[a] activity*
Hg^{2+}	0.01	107
	0.1	67
	1	55
	10	35
Mn^{2+}	1	71
Ag^{+}	1	73
K^{+}	1	100
Fe^{2+}	1	102
Co^{2+}	1	108
Mg^{2+}	1	110
Zn^{2+}	1	110
Ba^{2+}	1	117
Ca^{2+}	1	131

[a]Relative activity to a reference without ion.

TABLE V

INHIBITION OF XYLANASE BY SPECIFIC INHIBITORS

Inhibitor	*Concentration tested (mM)*	*Relative[a] activity*
N-Ethylmaleimide	20	100
N-Acetylimidazole	23	56
2-Hydroxy-5-nitrobenzyl bromide	10	0
EDTA[b]	50	92

[a]Relative activity to a reference without inhibitor. [b]Ethylenediaminetetra-acetic acid (disodium salt).

Action on larch 4-O-methylglucuronoxylan. — The larchwood xylan contained ~70% of xylose and 9% of 4-*O*-methylglucuronic acid, as determined by *m*-phenylphenol and carboxyl-reduction of the carbodiimide derivative, and had a d.p. of ~140, as measured by viscometry in methyl sulfoxide[24] (30°). Gymnosperm xylans are 4-*O*-methylglucuronoarabinoxylans containing 14–18% of 4-*O*-methylglucuronic acid attached to O-2, and 5–20% of arabinofuranose attached to O-3, of xylosyl residues of the main backbone. Arabinose, as well as glucomannan and glucan contaminants were absent from the larch-xylan samples used (Sigma, lots 128C-03641 and 122F-0302; Ega-Chemie, lot 3300649/3) contrary to the findings of other authors[20,25,26]. The purified larchwood-xylan samples still contained ~20% of polyphenolics, as demonstrated by the strong u.v. absorption at 270–280 nm after dissolution in acetyl bromide[27]. Furthermore, the xylan was faintly coloured, which could reflect the presence of lignin[28]. Delignification by such oxidising agents as sodium chlorite, which are known to partially depolymerise hemicelluloses[28], was therefore omitted. The occurrence of lignin in commercial xylans, either covalently linked or adsorbed, is usually unspecified in enzyme studies[7,20,25], but must be of some importance. It is possible that hydrophobic interaction between our xylanase and the lignin moiety could play a significant role in the enzyme–substrate interaction. The kinetic parameters were determined (Table II, Figs. 9 and 10) either in standard acetate buffer or in media containing various concentrations of methyl sulfoxide, a solvent for xylans[28]. The K_m value was 2.8 mg/mL in acetate buffer and decreased abruptly to ~1.3 mg/mL for 10% methyl sulfoxide. For 20–40% methyl sulfoxide, the value of K_m was stable at

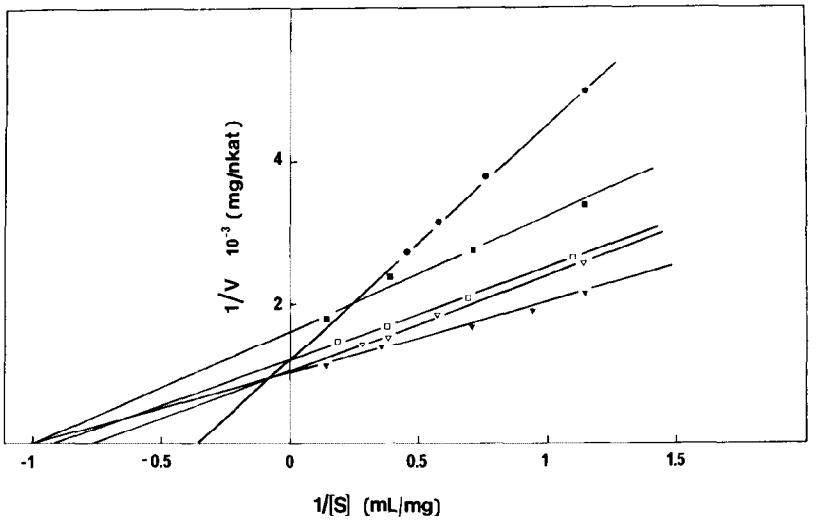


Fig. 9. Lineweaver–Burk plot for the xylanase acting on larch 4-*O*-methylglucuronoxylan (see Experimental): standard conditions (—●—), the enzyme in the presence of Me_2SO, 10% (—▽—), 20% (—▼—), 30% (—□--), and 40% (—■—).

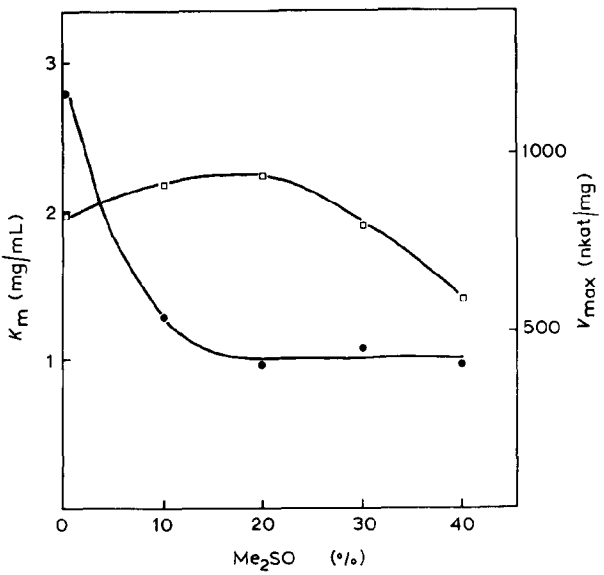


Fig. 10. Variation of K_m (—●—) and V_{max} (—□—) for the xylanase acting on larch 4-*O*-methylglucuronoxylan, as a function of Me_2SO percentage in the standard activity assay.

1 mg/mL, which corresponds to 0.7 mg/mL as (1→4)-β-D-xylosidic bonds. This figure is very close to the K_m of *Trametes hirsuta* xylanase for a very similar water-soluble 4-*O*-methylglucuronoxylan from willow (xylose–uronic acid ratio = 7.6:1). This greater affinity of *Irpex lacteus* xylanase for larch 4-*O*-methylglucuronoxylan in methyl sulfoxide than in water could be related to better solubilisation by the former solvent. This, in turn, could be related to the lack of activity of *Aspergillus niger* xylanase[20] on water-insoluble, commercial larch xylan (~40% of total commercial xylan). The maximum velocity V_{max} increased slightly up to 20% methyl sulfoxide and then decreased gradually as the percentage increased to 40%. Methyl sulfoxide above 20% therefore acts as a non-competitive inhibitor of the xylanase. No inhibition was observed with D-xylose (mM), L-arabinose (mM), and methyl β-D-xylopyranoside (mM and 10mM).

The hydrolysis of larch 4-*O*-methylglucuronoxylan by the xylanase was monitored by measuring the appearance of reducing sugars, using the Nelson procedure[29]. The reducing power of the medium reached a maximum at 24 h, which corresponded to cleavage of 27% of the (1→4)-β-D-xylosidic bonds. This figure is much smaller than that (49%) for the hydrolysis of willow xylan by *Trametes hirsuta* xylanase[30]. Termination of the reaction was not caused by denaturation or inhibition of the xylanase, because it retained its full activity after 55 h. The average d.p. of the oligosaccharides formed was 4.2, taking into account the neutral and acidic products. Analysis of the neutral components by h.p.l.c. is shown in Fig. 11.

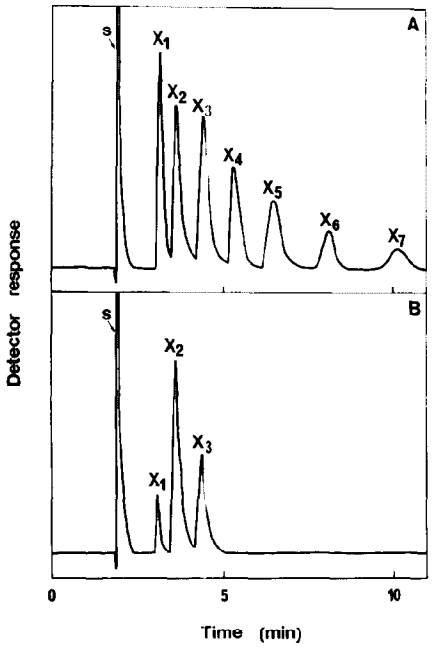


Fig. 11. H.p.l.c. (see Experimental) of *A*, (1→4)-β-D-xylo-oligosaccharides (X_1 = xylose, X_2 = xylobiose, *etc.*) obtained by partial hydrolysis of larchwood 4-*O*-methylglucuronoxylan with 0.03M trifluoroacetic acid[32]; *B*, xylanase hydrolysate.

Xylobiose was the major product, together with xylotriose and xylose; higher neutral homologues were detected, and thus the *Irpex lacteus* xylanase is concluded to be an endo-(1→4)-β-D-xylanase.

EXPERIMENTAL

Enzyme. — The enzyme was a commercial, crude preparation (Driselase; Kyowa Hakko Kogyo Co., Tokyo) of cell-wall polysaccharidases from the basidiomycetes *Irpex lacteus (Polyporus tulipiferae)*, which is generally used for protoplast preparation.

Substrates. — Commercial larchwood xylan (Sigma) was purified by barium chloride precipitation[31]. The content of neutral sugars was analysed by g.l.c. (on an SP-2340 column[32] at 225°) of the alditol acetate derivatives[33] obtained after hydrolysis[34] with 2M trifluoroacetic acid; acidic sugars were determined by the *m*-phenylphenol colorimetric method[35]. The nature of the acidic sugars was established by carboxyl-reduction of the carbodiimide derivatives[36] with $NaBH_4$, alditol acetate derivatisation, and g.l.c. The xylan contained one 4-*O*-methylglucuronic acid residue per eight xylosyl residues, according to the data of Aspinall and McKay[25]. Only ~80% of the dry matter of the purified xylan was identified as polysaccharidic

material; the remainder was probably polyphenolic, as qualitatively measured by the acetyl bromide procedure[27].

The other substrates used were carboxymethylcellulose sodium salt (Sigma C-4888, medium viscosity), avicel (Merck), cellulose azure (Calbiochem 219481), arabinan (Koch–Light 52867), larchwood arabinogalactan (Sigma A-2012), polygalacturonic acid (ICN 102771), yeast mannan (Sigma M-7504), dextran (Sigma D-4751), lichenan from *Cetraria islandica* (Sigma L-8378), laminarin from *Laminaria* spp. (Fluka 61400), waxy-maize beta-limit dextrin (gift of Dr. P. Colonna), white-lupin acidic galactan (prepared in our laboratory), *p*-nitrophenyl α-D-glucopyranoside, α-D-galactopyranoside, β-D-galactopyranoside, α-L-arabinofuranoside, and α-L-fucopyranoside, and *o*-nitrophenyl β-D-xylopyranoside (Sigma).

Enzyme assay. — Xylanase activity was determined by mixing enzyme solution (5–200 μL, <2 nkat) with 0.6 mL of a 1% dispersion of xylan in 0.1M acetate buffer (pH 4.2). The volume was made up to 1.2 mL with distilled water. After incubation for 20 min at 40°, liberated reducing sugars were measured by the Somogyi–Nelson procedure[29], using D-xylose as the standard; 1 nkat of enzyme liberated 1 nmol of xylose-equivalent reducing power per second. Other polysaccharidases were measured under similar conditions (3 nkat), but in 0.05M acetate buffer (pH 5.0) at 30° for 15 h.

Glycosidases were determined by incubating[37] 0.6 mL of nitrophenyl glycoside (0.2%) in 0.05M acetate buffer (pH 5.0) with 0.6 mL of enzyme solution (3 nkat) at 30° for 15 h. Liberation of *p*- and *o*-nitrophenol was determined by adding 0.2 mL of reaction medium in 0.6 mL of aqueous 20% sodium carbonate, and measuring the absorbance at 400 nm for *p*-nitrophenol and at 414 nm for *o*-nitrophenol.

Protein determination. — Protein was recorded in column effluents at 280 nm. Protein was also measured by the Lowry procedure[38], using bovine serum albumin (Sigma, Fraction V, A-4503) as reference standard.

General methods. — Polysaccharides were hydrolysed[34] in 2M trifluoroacetic acid for 1.25 h at 120°. The glycosidic moiety of purified xylanase (1 mg) was hydrolysed by boiling in M hydrochloric acid for 1 h[39]. Sugars were reduced with sodium borohydride, and the alditols were acetylated with acetic anhydride–pyridine (1:1) at 120° for 20 min[33,40]. G.l.c. of alditol acetates was performed[32] on a glass column (180 × 2 mm i.d.) packed with 3% of SP-2340 on 100–120 mesh Supelcoport (Supelco) at 225°. Products of the enzymic hydrolysis of larchwood xylan were separated by h.p.t.l.c. with acetonitrile–water (85:15). Sugars were detected by the diphenylamine–aniline reagent[41].

Electrophoresis and isoelectric focusing. — SDS-polyacrylamide gel electrophoresis[42] was performed by using a 10–20% acrylamide gradient gel at pH 8.2. Protein bands were detected by staining with Coomassie Brilliant Blue G. The molecular weight of the purified xylanase was determined by comparison with a calibration kit (Pharmacia) for low molecular weight protein.

The isoelectric focusing was performed by using an LKB 2117 Multiphor device, with polyacrylamide plates[43] containing ampholine of pH range 8.0–10.5 (Pharmacia).

Amino acid determination. — The analysis of amino acids was performed by Dr. M. A. O'Neill, using the heptafluorobutyric *n*-propyl derivatisation method[44] on a dialysed and freeze-dried sample (200 μg) of the homogeneous xylanase.

Glycoprotein sugar analysis. — Carbohydrates present in the xylanase were measured by the phenol–sulfuric acid technique[45] on a desalted and vacuum-concentrated enzyme solution, with D-glucose as the standard.

H.p.l.c. — Samples of xylo-oligosaccharides obtained by enzymic hydrolysis were analysed with a Waters Associates chromatograph equipped with a differential refractometer and a column (250 × 4.6 mm i.d.) packed with Lichrosorb NH2 (particle size, 5 μm) (Merck); elution was effected with acetonitrile–water (70:30) at 2 mL/min. Prior to analysis, samples were deionised with Dowex 1-X8 ($HCOO^-$) and Amberlite IR-120 (H^+) resins, and concentrated *in vacuo* at 40°. The sample size was 25 μL.

Enzyme purification. — Crude Driselase (5 g) was stirred with 0.1M phosphate buffer (25 mL, pH 7.0) for 30 min at 4°. Insoluble material was removed by centrifugation at 40,000*g* for 10 min (4°), and the supernatant solution was desalted by using a column (2.2 × 68 cm) of Sephadex G25 M equilibrated with 0.1M phosphate buffer (pH 7.0).

Chromatography. — (a) On DEAE-Sepharose CL-6B. A column (3.2 ×18 cm) of DEAE-Sepharose CL-6B was equilibrated with 10mM phosphate buffer (pH 7.0). A sample (66 mL) of the desalted enzyme was dialysed against 10mM phosphate buffer (pH 7.0) and applied to the column at 160 mL/h. The xylanase activity remained unbound, whereas most of the coloured material was bound to the anion exchanger.

(b) On CM-Sepharose CL-6B. A column (3.2 × 13 cm) of CM-Sepharose CL-6B was equilibrated with 20mM acetate buffer (pH 4.4). A sample (158 mL) of the fraction not retained on DEAE-Sepharose CL-6B was dialysed overnight against mM phosphate buffer (pH 7.0) and then against 20mM acetate buffer (pH 4.4) for 2.5 h at 4°, and finally applied to the column at 150 mL/h. After elution with 500 mL of the starting buffer, a 0→0.22M linear gradient of sodium chloride (700 mL) in the same buffer was applied. The chromatogram is shown in Fig. 1.

(c) On Phenyl-Sepharose CL-4B. Fractions from (*b*) eluted in the range 0.15–0.20M were combined (196 mL), concentrated by dialysis in the presence of Sephadex G 200, dialysed overnight against 10mM phosphate buffer (pH 7.0), diluted with an equal volume of cold 10mM phosphate buffer (pH 7.0) containing 2M ammonium sulphate, applied to a column (0.9 × 22 cm) of Phenyl-Sepharose CL-4B equilibrated with 10mM phosphate buffer (pH 7.0) containing M ammonium sulphate, and eluted with the starting buffer (22 mL/h) for ~2 column volumes. A linear gradient (0→50%) of aqueous ethylene glycol (300 mL) was then applied

with a simultaneous decrease of ammonium sulphate from M→0. The chromatogram is shown in Fig. 2.

(d) On Ultrogel AcA54. — Fractions from (*c*) eluted by the 20–30% range of the ethylene glycol gradient were combined (100 mL), dialysed overnight against 50mM Tris-HCl buffer (pH 7.2) containing 0.15M sodium chloride, concentrated by dialysis to 7.8 mL, applied to a column (1.6 × 87 cm) of Ultrogel AcA54 equilibrated with 50mM Tris-HCl buffer (pH 7.2) containing 0.15M sodium chloride, and eluted with the starting buffer at 11 mL/h. The resulting chromatogram is shown in Fig. 3.

(e) On Concanavalin A-Ultrogel AcA22. — Active fractions from (*d*) (elution volume, 110–135 mL) were combined (26 mL), concentrated, dialysed against 50mM Tris-HCl buffer (pH 7.2) containing 0.1M sodium chloride and 0.1mM manganese chloride, added to a column (0.9 × 15 cm) of ConA–Ultrogel AcA22 (from IBF) equilibrated with 50mM Tris-HCl buffer (pH 7.2) containing 0.1M sodium chloride and 0.1mM manganese chloride, and eluted (45 mL/h) for ~4 column volumes. A linear gradient (0→0.1M) of methyl α-D-mannopyranoside (100 mL) in the starting buffer was then applied. The chromatogram is shown in Fig. 4.

Properties. — *(a) pH Optimum.* The xylanase activity was measured under standard conditions by suspending xylan in citrate–phosphate buffer of various pH values, instead of 0.1M acetate buffer, pH 4.2 (Fig. 5).

(b) pH Stability. The xylanase (6 nkat) was incubated in universal buffer of various pH values at 30°. After 17 h, the enzyme samples were dialysed against mM phosphate buffer (pH 7.0), and the residual xylanase activity was measured under standard conditions (Fig. 8).

(c) Temperature optimum. The xylanase activity was measured under standard conditions in the temperature range 20–80°. The medium was heat-stabilised for 5 min before adding the enzyme (Fig. 6).

(d) Temperature stability. The enzyme (6 nkat) in 0.1M acetate buffer (pH 4.2) was maintained at various temperatures (4–80°) for 0.5 h, then the residual activity was measured under standard conditions (Fig. 7).

(e) Kinetic parameters. The activity of the purified xylanase was measured under standard conditions with variation of the final concentration of larchwood xylan from 0.4 to 18 mg/mL. For concentrations >7 mg/mL, the mixtures associated with Nelson colorimetric assays were centrifuged prior to reading the absorbance at 520 nm, because of the high turbidity due to the xylan. The influence of substrate solubility on K_m and V_{max} was tested by measuring initial velocities under the following conditions. A solution of larchwood xylan in methyl sulfoxide at 20° was diluted with water to give methyl sulfoxide concentrations from 10–40% in standard assays (final xylan concentrations, 0.4–7 mg/mL). Methyl sulfoxide did not interfere in the Nelson assay. The data were expressed by the Lineweaver–Burk reciprocal-plots method (Figs. 9 and 10).

(f) Effect of metal ions. The xylanase activity was assayed under standard

conditions in the presence of mM metal ions. The effect of metal ions on the Nelson assay was preliminarily tested (Table IV).

(g) Influence of specific inhibitors. The xylanase (7 nkat) was incubated for 60 min at 20° with the following inhibitors: *N*-ethylmaleimide (20mM in 0.1M phosphate buffer, pH 7.0), *N*-acetylimidazole (23mM in 10mM Tris-HCl buffer, pH 7.5), 2-hydroxy-5-nitrobenzyl bromide (10mM in 0.1M acetate buffer, pH 4.4, in the dark), and EDTA (50mM in 10mM phosphate buffer, pH 7.0). After dialysis for 24 h against 10mM phosphate buffer (pH 7.0), the residual activity was measured under standard conditions with an appropriate blank (Table V).

Hydrolysis of the larchwood xylan by the homogeneous xylanase. — Larchwood xylan (466 mg) was suspended with stirring in 50mM acetate buffer (50 mL, pH 5.0) containing 0.02% of NaN_3 at 40°. At zero time, homogeneous xylanase solution (300 μL, ~30 nkat) was added. After 55 h, when the reducing power of the medium had become constant, the mixture was heated at 100° for 5 min and then centrifuged, and the supernatant solution was diluted with ethanol to a final concentration of 40%. After centrifugation, the supernatant solution was evaporated to dryness *in vacuo* at 40°. A solution of the residues in distilled water was deionised with Dowex 1-X8 ($HCOO^-$) and Amberlite IR-120 (H^+), and then concentrated *in vacuo* prior to h.p.t.l.c. and h.p.l.c. (Fig. 11).

ACKNOWLEDGMENTS

The authors thank J. Vigouroux for technical assistance, Drs. R. R. Selvendran and M. A. O'Neill (Food Research Institute, Norwich, Great Britain) for the amino acid analysis, Professor J.-P. Joseleau (CERMAV, Grenoble, France) for identification of 4-*O*-methylglucuronic acid, and Dr. Y. Bertheau (Laboratoire de Pathologie Végétale, INRA-INA, Paris, France) for helpful discussions about the electrofocusing procedure.

REFERENCES

1 R. F. H. Dekker and W. A. Lindner, *South Afric. J. Sci.*, 75 (1979) 65–71.
2 G. O. Aspinall, in J. Preiss (Ed.), *The Biochemistry of Plants*, Academic Press, New York, 1980, pp. 473–500.
3 J.-P. Joseleau, in B. Monties (Ed.), *Les Polymères Végétaux*, Gauthier–Villars, Paris, 1980, pp. 87–121.
4 K. C. B. Wilkie and S. L. Woo, *Carbohydr. Res.*, 57 (1977) 145–162.
5 R. F. H. Dekker and G. N. Richards, *Adv. Carbohydr. Chem. Biochem.*, 32 (1976) 277–352.
6 J. Comtat and J.-P. Joseleau, *Carbohydr. Res.*, 95 (1981) 101–112.
7 C. J. Baker, C. H. Whelan, and D. F. Bateman, *Phytopathology*, 67 (1977) 1250–1258.
8 K. Nisizawa, *J. Biochem. (Tokyo)*, 42 (1955) 825–835.
9 K. Nisizawa and Y. Hashimoto, *Arch. Biochem. Biophys.*, 81 (1959) 211–222.
10 K. Nisizawa, I. Morimoto, N. Handa, and Y. Hashimoto, *Arch. Biochem. Biophys.*, 96 (1962) 152–157.
11 T. Kanda, K. Wakabayashi, and K. Nisizawa, *J. Ferment. Technol.*, 48 (1970) 607–615.
12 T. Kanda, K. Wakabayashi, and K. Nisizawa, *J. Biochem. (Tokyo)*, 79 (1976) 977–988.
13 T. Kanda, K. Wakabayashi, and K. Nisizawa, *J. Biochem. (Tokyo)*, 79 (1976) 989–995.

14 T. KANDA, K. WAKABAYASHI, AND K. NISIZAWA, *J. Biochem. (Tokyo)*, 79 (1976) 997–1006.
15 T. KANDA, S. NAKAKUBO, K. WAKABAYASHI, AND K. NISIZAWA, *J. Biochem. (Tokyo)*, 84 (1978) 1217–1226.
16 T. KANDA, K. WAKABAYASHI, AND K. NISIZAWA, *J. Biochem. (Tokyo)*, 87 (1980) 1625–1634.
17 K. E. ERIKSSON AND B. PETTERSSON, *Int. Biodeterior. Bull.*, 7 (1971) 115–119.
18 I. V. GORBACHEVA AND N. A. RODIONOVA, *Biochim. Biophys. Acta*, 484 (1977) 79–93.
19 M. G. PAICE, L. JURASEK, M. CARPENTER, AND L. B. SMILLIE, *Appl. Environ. Microbiol.*, 36 (1978) 802–808.
20 M. M. FREDERICK, J. R. FREDERICK, A. R. FRATZKE, AND P. J. REILLY, *Carbohydr. Res.*, 97 (1981) 87–103.
21 L. L. SO AND I. J. GOLDSTEIN, *J. Biol. Chem.*, 243 (1968) 2003–2007.
22 K. SUMIZU, M. YOSHIKAWA, AND S. TANAKA, *J. Biochem. (Tokyo)*, 50 (1961) 538–543.
23 M. KUBACKOVA, S. KARACSONYI, L. BILISICS, AND R. TOMAN, *Folia Microbiol. (Prague)*, 23 (1978) 202–209.
24 V. E. HUSEMANN, *J. Prakt. Chem.*, 155 (1940) 13–64.
25 G. O. ASPINALL AND J. E. MCKAY, *J. Chem. Soc.*, (1958) 1059–1066.
26 R. F. H. DEKKER AND G. N. RICHARDS, *Carbohydr. Res.*, 42 (1975) 107–123.
27 I. M. MORRISON, *J. Sci. Food Agric.*, 23 (1972) 455–463.
28 T. E. TIMELL, *Adv. Carbohydr. Chem.*, 19 (1964) 247–302.
29 N. NELSON, *J. Biol. Chem.*, 153 (1944) 375–380.
30 M. KUBACKOVA, S. KARACSONYI, AND R. TOMAN, *Folia Microbiol. (Prague)*, 21 (1976) 28–35.
31 L. TAIZ AND W. A. HONIGMAN, *Plant Physiol.*, 58 (1976) 380–386.
32 J.-M. BRILLOUET, J.-P. JOSELEAU, J.-P. UTILLE, AND D. LELIEVRE, *J. Agric. Food Chem.*, 30 (1982) 488–495.
33 J. F. SAWARDEKER, J. H. SLONEKER, AND A. JEANES, *Anal. Chem.*, 37 (1965) 1602–1604.
34 P. ALBERSHEIM, D. J. NEVINS, P. D. ENGLISH, AND A. KARR, *Carbohydr. Res.*, 5 (1967) 340–345.
35 N. BLUMENKRANTZ AND G. ASBOE-HANSEN, *Anal. Biochem.*, 54 (1973) 484–489.
36 R. L. TAYLOR AND H. E. CONRAD, *Biochemistry*, 11 (1972) 1383–1388.
37 F. G. J. VORAGEN, R. HEUTINK, AND V. PILNIK, *J. Appl. Biochem.*, 2 (1980) 452–468.
38 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
39 J. H. PAZUR, Y. TOMINAGA, L. S. FORSBERG, AND D. L. SIMPSON, *Carbohydr. Res.*, 84 (1980) 103–114.
40 R. R. SELVENDRAN, J. F. MARCH, AND S. G. RING, *Anal. Biochem.*, 96 (1979) 282–292.
41 J. L. BUCHAN AND R. I. SAVAGE, *Analyst (London)*, 77 (1952) 401.
42 U. K. LAEMMLI, *Nature (London)*, 227 (1970) 680–685.
43 A. WINTER, C. EK, AND U. B. ANDERSSON, *LKB Application Note*, 250 (1977) 1–13.
44 J. F. MARCH, *Anal. Biochem.*, 69 (1975) 420–442.
45 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–355.