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PURIFICATION AND CHARACTERIZATION OF A XYLOBIOSE- AND XYLOSE-PRODUCING ENDO-XYLANASE FROM *Aspergillus niger*

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ABSTRACT

A xylanase from a commercial *Aspergillus niger* pentoglycanase was purified to homogeneity by column chromatography on Ultrogel AcA 54, SP-Sephadex, Sephadex G-50, and SP-Sephadex. The enzyme hydrolyzed xylotriose slowly to xylose and xylobiose, and xylotetraose and higher xylo-oligosaccharides rapidly to mixtures of smaller xylo-oligosaccharides, with xylobiose and xylose being the preponderant final products. The anomeric configuration of the products was inverted, in contrast to the behavior of most other carbohydrases that initially produce mixtures of oligosaccharides. This enzyme is a glycoprotein having an amino acid composition high in acidic residues. Its molecular weight is 20,800 and its isoelectric point is at pH 6.7. Optimal pH values for activity and stability are between 4 and 6 and, in a 20-min assay, maximal activity is attained at 55°.

INTRODUCTION

Although enzymes that break down xylan have been studied for approximately 30 years, little is known of their identity or of the mechanisms involved in breakdown of xylan. This is mainly because many xylanases take part in a synergistic relationship and few xylanases have been purified to homogeneity.

Recently, however, reports of homogeneous xylanases have issued from a number of laboratories. Various enzymes that produce primarily xylobiose and higher xylo-oligosaccharides, and therefore are grouped together under EC 3.2.1.8 [(1→4)-β-D-xylan xylanohydrolase], have been isolated from cultures of *Aspergillus*[1–4], *Schizophyllum*[5,6], *Stereum*[7], *Trichoderma*[2,8,9], *Bacillus*[10], *Penicillium*[11], *Ceratocystis*[12], *Cephalosporium*[13], *Oxiporus*[2], *Trametes*[14], and *Streptomyces*[15].

Although many strains produce a large number of xylanases having apparently similar or complementary activities, in only one case[4] has the xylanase complex from one of these strains been studied in sufficient detail to determine the roles of most of its members. Though most of the homogeneous xylanases reported produce large amounts of xylobiose, there is no unequivocal evidence that any enzyme whose major product is xylobiose acts by an exo mechanism, attacking the chain from the nonreducing end, with the configuration of the product being inverted and reaction

rate increasing with increasing chain-length[16]. This gap appears unusual, as exo-hydrolases that produce primarily xylose from xylan or aryl β-D-xylosides are apparently elaborated by *Bacillus pumilus*[17,18], *Malbranchea pulchella*[19], and possibly by *Aspergillus niger*[20], while exo-hydrolases that yield products of various lengths are found in the amylase family, and those that produce dimers play a large role in the cellulase family.

We have been purifying to homogeneity xylanases from a commercial preparation of *A. niger* hemicellulase, and subjecting each to extensive characterization, including determination of molecular weight, amino acid profile, carbohydrate content, product profile, and effect of temperature and pH on activity and stability, to differentiate them from each other and to understand better how they work together. This paper is the first in a series on these findings. It reports the purification and properties of a xylanase that catalyzes the final production of large amounts of xylobiose and xylose from xylan, following the production of xylo-oligosaccharides of various lengths.

EXPERIMENTAL

Enzyme assay. — Enzyme activity was determined with a Somogyi–Nelson reducing-sugar assay[21]. Samples (20–100 μL) of enzyme solution were mixed with 0.25 mL of ~1% xylan (2% of Sigma X-3875 larchwood xylan, Lots 97C-0066-1 or 125C-00582, dissolved in water at room temperature and the undissolved material centrifuged out), and 0.75 mL of 0.075M sodium acetate buffer at pH 4.8 for enzyme separations or pH 5.0 for enzyme characterization. The mixture was incubated for 20 min at 40°, and the absorbance of the final solution in a cuvette of 1-cm path length was determined at 500 nm.

Activity was measured in units (U), where 1 U is the amount of enzyme that releases reducing power equivalent to 1 μmol of xylose in 1 min under the conditions of the assay.

Protein determination. — With column effluents, protein was determined by measuring the absorbance at 280 nm of solution in a cuvette of 1-cm path length. With enzyme pools, the Bio-Rad protein assay, based on Bradford's method[22], was used with bovine serum albumin as standard.

Isoelectric focusing and electrophoresis. — The isoelectric focusing procedure followed that of Righetti and Drysdale[23], with duplicate 100-mm long, 5-mm i.d. gels. LKB Ampholine 3/10 ampholyte was employed. After ~5.5 h at a constant power of 0.167W/tube, when the voltage had reached ~450 V, one gel of each sample was sliced into 1.5-mm lengths. These were extracted with 1-mL portions of water during 2 days at 4°, with 100-μL aliquots being assayed for xylanase activity with a 120-min incubation at 40° and the rest being tested for pH. The duplicate gel was stained with Coomassie Brilliant Blue R to determine protein bands.

Disc poly(acrylamide)-gel electrophoresis was performed by the method of Laemmli[24].

High-pressure liquid chromatography. — Samples of xylosides after enzymic hydrolysis were analyzed by differential refractometry with a Waters ALC 201 liquid chromatograph, using an Aminex HPX-42 cation-exchange column (7.8 mm i.d. and 300 mm length) kept at 85°. The sample size was 100 μL, and the flow rate 0.3 mL/min.

Product configuration. — Homogeneous, desalted xylanase was incubated at 40° with fully mutarotated xylotetraose. Samples were quick-frozen in a Dry Ice–methanol bath, lyophilized, and dissolved in Pierce Tri-Sil "Z" [1.5 meq/mL *N*-(trimethylsilyl)imidazole in dry pyridine][25]. Aliquots (2 μL) were injected into a Varian model 3700 gas chromatograph equipped with a flame-ionization detector and a 1.5-m long, 6.2-mm o.d. glass column packed with 3% of SE-30 on 100–120 mesh Gas Chrom Q. After 1 min at 160°, the column temperature was increased by 10°/min to 300°, where it was maintained until the silylated xylotetraose was eluted. The identities of the α and β peaks was confirmed with samples of trimethylsilylated α- and β-D-xylose.

Molecular-weight determination. — The molecular weight of the purified enzyme was measured by adding a 2.5-mL portion to a column (15 mm i.d. and 1 m long) of Sephadex G-50 Superfine, eluted with 0.25M sodium acetate buffer, pH 5, and 0.05M sodium chloride at 0.25 mL/min, and determining the absorbance of eluate samples by the regular assay.

Standards of 2.5 mL in 1 mg/mL concentration were similarly chromatographed and the peaks were determined by absorbance at 280 nm.

Amino acid determination. — A desalted, homogeneous enzyme-sample containing ~50 μL of protein was hydrolyzed with 6M hydrochloric acid, vacuum-evaporated to dryness, dissolved in 0.2M citrate buffer at pH 2.2, split into two parts, and each part injected into a Durrum D-400 amino acid analyzer.

Glycoprotein carbohydrate analysis. — Purified enzyme was desalted, concentrated by evaporation to 4 mL, and dialyzed through cellulose tubing against four 200-mL changes of water for 1.5 h each. The carbohydrate composition was then measured by the phenol–sulfuric acid method[26]. Protein was determined as before, except that ovalbumin was used as standard.

Materials. — The source of enzyme was Rohm and Haas Rhozyme HP-150 Concentrate, lot 3-0007, a pentoglycanase obtained from *A. niger*.

For specificity studies, both the soluble fraction and the approximately half of the xylan that remained undissolved in a 2% aqueous mixture were used. Also prepared were soluble and insoluble fractions whose arabinosyl-initiated side-chains had been removed by acid hydrolysis. Xylan (50 g of Sigma lot 97C-0066-1) was added to 4795 mL of water and the undissolved material (~20 g) was centrifuged out. Both the soluble and insoluble portions were treated with sulfuric acid at pH 2 for 90 min at 100°. The soluble fraction was made neutral with barium hydroxide and the precipitate removed by centrifugation before use. The insoluble fraction was treated with sodium hydroxide and washed with water.

Compositions of polysaccharides were determined by modifications of the method of Selvendran *et al.*[27]. Mixtures of polysaccharide (25 mg) and 2M trifluoroacetic acid (10 mL) were incubated at 100° for various times up to 4 h. Samples were

filtered and twice vacuum-evaporated. After being redissolved in water a second time, compositions of the hydrolyzate were measured by l.c. The hydrolyzate from soluble Sigma xylan had 48% xylose, 13% glucose, 10% arabinose, and 29% glucuronic acid and unhydrolyzed polymer. The hydrolyzate from insoluble Sigma xylan was 71% xylose, 10% glucose, 11% arabinose, and 8% glucuronic acid and unhydrolyzed polymer. Soluble larchwood xylan (Koch–Light lot 74025) was 62% xylose, 8% glucose, 13% arabinose, and 17% glucuronic acid and unhydrolyzed polymer. Insoluble xylan (Koch–Light) had 69% xylose, 11% glucose, 10% arabinose, and 10% glucuronic acid and unhydrolyzed polymer. Pectin (Sigma P-9135, lot 64C-0028) had ~10% xylose, whereas galacturonan (Sigma P-3889, lot 65C-0192) contained ~8% xylose.

Xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), and a mixture of xylopentaose (X_5) to xylononaose (X_9) were prepared by hydrolysis of xylan with 1% sulfuric acid for 90 min at 100°, following treatment with glucoamylase to remove contaminating starch. The acid hydrolyzate was passed through a column[28] of 50% Darco G-50–50% Celite 560, using a 1-butanol–water linear gradient that reached 3.5% 1-butanol. Acid hydrolyzates of portions of each product contained only xylose as determined by liquid chromatography.

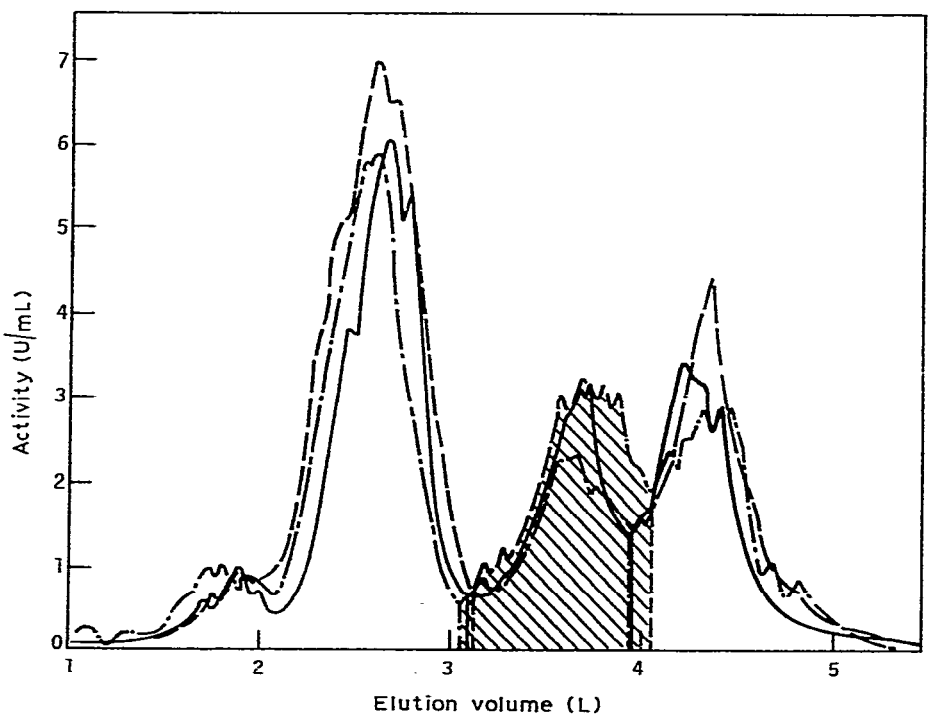


Fig. 1. Elution patterns of crude hemicellulase at 119 mL loading and 1.9 mL/min from a column (51 mm i.d. and 1430 mm) of Ultrogel AcA 54 at pH 4.5.

ENZYME PURIFICATION

The xylanase described in this paper was purified to homogeneity by adding 50 g of crude enzyme to 0.025M sodium acetate buffer at pH 4.5 to make 360 mL. The slurry was stirred for 30 min at 25° and for 2.5 h at 4°, the times being limited to minimize protease attack. After centrifugation, three equal portions of the supernatant solution were added to a column of Ultrogel AcA 54 kept at 4°, as were all enzyme-separation columns in this work, and eluted with the buffer already described (Fig. 1). The first peak represents amylase attack on a starch contaminant in the xylan used in the assay. The other two peaks exhibit mainly xylanase activity.

The hatched peaks from all three runs were pooled and applied to a column of SP-Sephadex C-25 eluted with the buffer already described. After two column volumes had been collected, a linear gradient of sodium chloride, with the high-salt side containing 0.035M sodium chloride dissolved in buffer, was applied (Fig. 2).

Two 100-mL samples of the hatched pool from the previous step were evaporated at room temperature to 25 and 31 mL and added separately to a column of Sephadex G-50 Superfine. After elution with 0.3M citrate buffer at pH 5.1 containing 0.04M sodium chloride, one peak showing xylanase activity was collected from each run (Fig. 3).

These two peaks, containing 50 and 68 mL, were pooled, titrated to pH 4.5 with hydrochloric acid, diluted ten-fold, and added to a column of SP-Sephadex G-25,

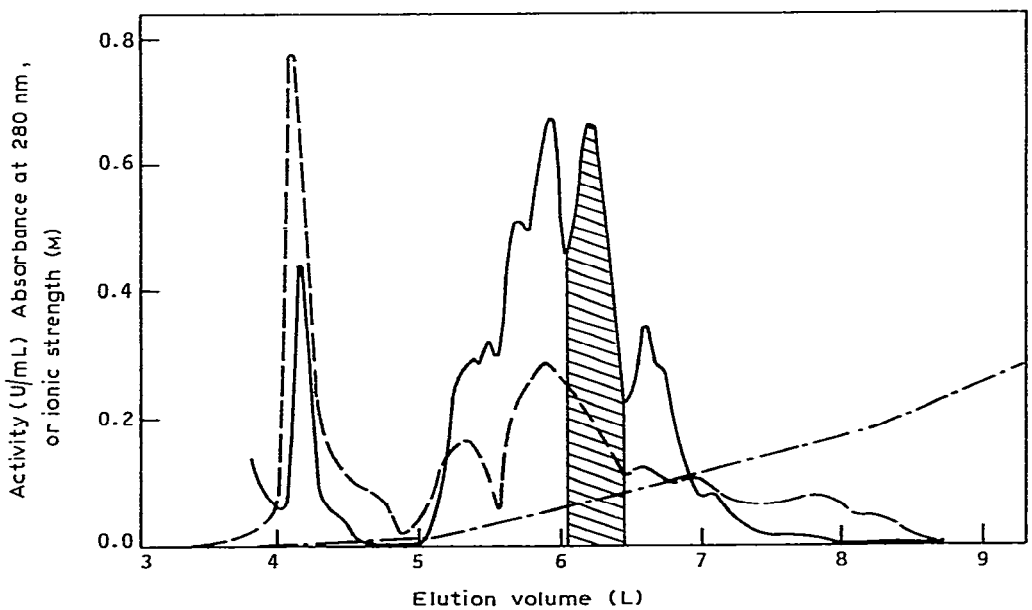


Fig. 2. Elution pattern of the feed from the Ultrogel AcA 54 separation at 2805 mL loading and 1.0 mL/min from a column (33 mm i.d. and 640 mm) of SP-Sephadex C-25 at pH 4.5. Activity (———), absorbance at 280 mm (– – – – –), and ionic strength (–·–·–).

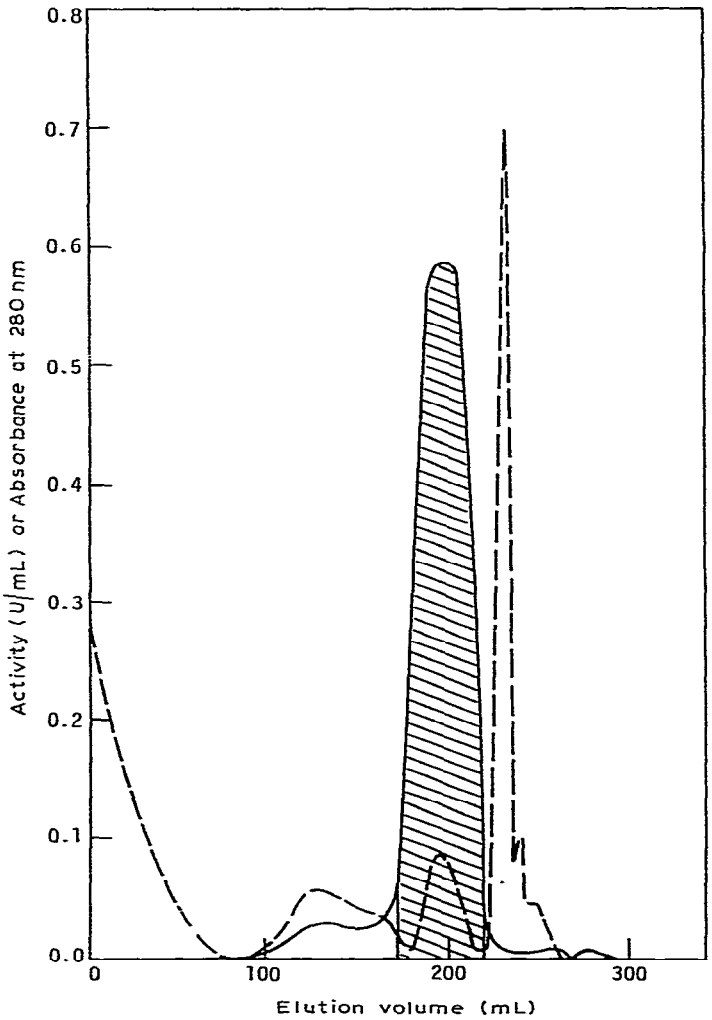


Fig. 3. Elution pattern of the feed from the SP-Sephadex separation at 25 mL loading and 0.2 mL/min from a column (22 mm i.d. and 1330 mm) of Sephadex G-50 Superfine at pH 5.1. Curves are as identified in Fig. 2.

eluted with 0.025M acetate buffer, pH 4.6, with a linear gradient constructed with 0.3M sodium chloride dissolved in buffer on the high side (Fig. 4).

The 76-mL heart cut was pooled and found to be homogeneous by isoelectric focusing and by disc-gel electrophoresis at pH 8.8 (Figs. 5A–5C). The isoelectric point was at pH 6.7. Chromatography on Sephadex G-50 Superfine to determine the molecular weight of this preparation yielded a single, sharp, symmetrical peak of activity with no shoulders.

The complete purification of the enzyme, from crude preparation to homogeneous xylanase, is presented in Table I. As 22.4% of the activity was chosen for further processing after the Ultrogel AcA 54 step, 25.7% was brought forward after the first

TABLE I

PURIFICATION OF XYLANASE TO HOMOGENEITY

Step	*Volume (mL)*	*Activity (U/mL)*	*Protein concentration (mg/mL)*	*Specific activity (U/mg)*	*Total activity (U)*	*Yield (%)*	*Cumulative yield (%)*	*Purification*	*Cumulative purification*
Crude enzyme	360	67.2	11.1	6.05	24,000	100	100	1	1
Centrifugation	357	64.2	12.2	5.28	22,900	94.7	94.7	0.873	0.873
Chromatography on Ultrogel AcA 54	2805	1.68			4,720	20.6	19.5		
Chromatography on SP-Sephadex C-25	417	0.46	0.021	21.9	192	4.06	0.793	4.15	3.62
Evaporation (200-mL aliquot)	56	1.82	0.083	21.9	102	111	0.878	1.00	3.62
Chromatography on Sephadex G-50	118	0.85	0.021	40.5	100	98.4	0.864	1.85	6.69
Chromatography on SP-Sephadex C-25	76	0.63	0.011	57.3	47.9	47.8	0.413	1.42	9.47

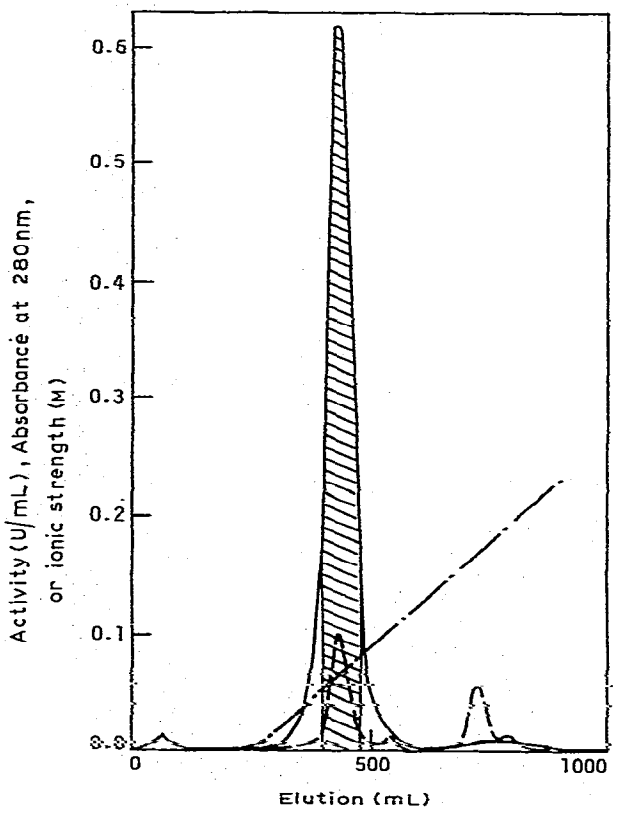


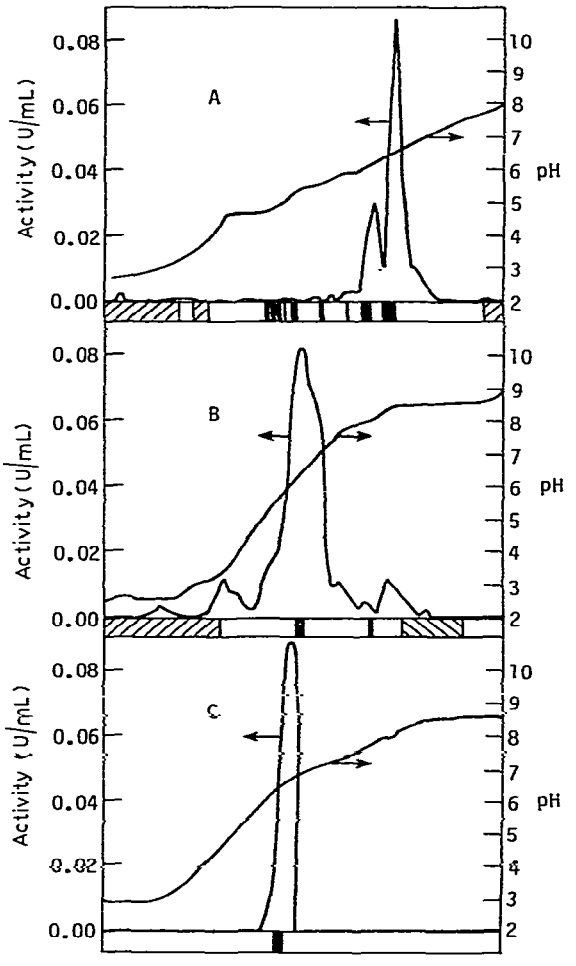
Fig. 4. Elution pattern of the feed from the Sephadex G-50 separation at 1180 mL loading and 1 mL/min from a column (17 mm i.d. and 590 mm of SP-Sephadex C-25 at pH 4.6. Curves are as identified in Fig. 2.

separation on SP-Sephadex, and 90.4 and 83.6% were pooled from the Sephadex G-50 and the second SP-Sephadex steps, respectively; the cumulative yield of this xylanase, based on what was pooled, was ~9.5%.

PROPERTIES OF HOMOGENEOUS XYLANASE

Specificity. — Soluble Sigma xylan, both untreated and treated to remove arabinosyl-initiated branches, was hydrolyzed extensively in the regular assay by homogeneous xylanase. There was no activity on untreated insoluble Sigma xylan and only slight activity on debranched, insoluble xylan.

To investigate more fully the hydrolysis of xylan, 13 mL of a 2% suspension of Koch–Light xylan was mixed with 1 mL of 0.3M acetate buffer, pH 5.5, and 0.2 mL of 1% sodium azide. This mixture was incubated at 40° under shaking at 300 r.p.m. with 1 mL of homogeneous xylanase. Samples withdrawn at specified times were



Figs. 5A, 5B, and 5C. Activities and protein stains of gels after isoelectric focusing of eluates from initial SP-Sephadex, Sephadex G-50, and final SP-Sephadex column chromatography.

analyzed by l.c. with a column of Bio-Rad HPX-42 preceded by a guard column containing Bio-Rad 991–1143, which removed the enzyme.

Initially, only a single peak, that of xylan, appeared on l.c. analysis, but by the first sample (taken at 5 min) after addition of enzyme, appreciable amounts of oligosaccharides had appeared (Fig. 6). On a molar basis, the concentration of xylobiose did not exceed that of trisaccharide and tetrasaccharide until almost 2 h had elapsed. The molar concentration of xylose was less than that of tri- and tetrasaccharides for an extended period. Longer oligosaccharides were not fully removed, as they were when linear xylo-oligosaccharides were hydrolyzed. Neither L-arabinose nor any uronic acid monomer was found at any time.

On a weight basis, this enzyme hydrolyzed slightly over 75% of the soluble xylan to molecules of 6 saccharide units or less.

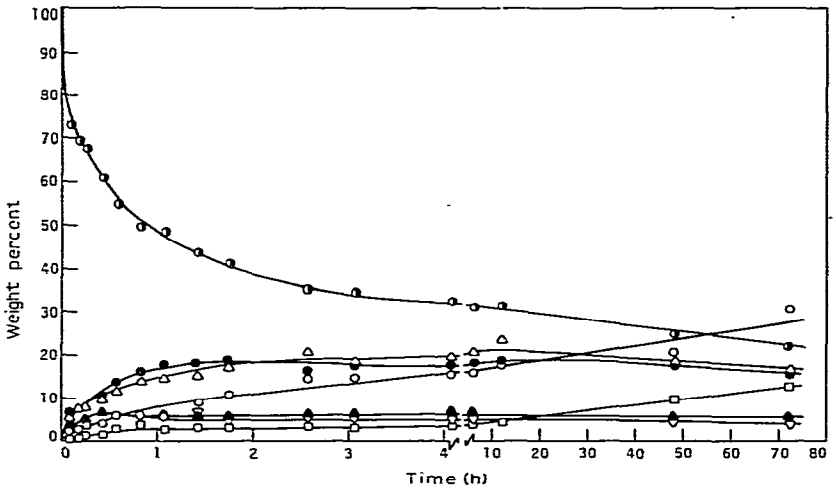


Fig. 6. Hydrolysis of 2% larchwood xylan by homogeneous xylanase. Product concentrations were calculated after the buffer and sodium azide peak-areas had been discarded. Degree of polymerization: 1(□), 2(○), 3(△), 4(●), 5(▼), 6(▽), and >6(◑).

Of the 0.5% of xylosides subjected to attack for various times in the regular assay with equal activities of homogeneous xylanase, xylobiose was totally resistant, whereas X_3 was hydrolyzed slowly to X_2 and xylose. Xylotetraose was broken down to X_2, with some X_3 and xylose, and the X_3 was more slowly hydrolyzed to X_2 and xylose. An X_5–X_9 mixture was rapidly hydrolyzed to a mixture of shorter xylo-oligosaccharides, chiefly X_3, X_2, and X_4, with X_2 being the predominant final product.

The homogeneous enzyme had little or no activity in a 60-min assay on arabinogalactan, CM-cellulose, crystalline cellulose, chitin, dextran, "dextrin", glucomannan, inulin, laminaran, locust-bean gum, yeast mannan, or *p*-nitrophenyl β-D-glucoside. There was some activity on untreated cellulose (Schleicher and Schüll

TABLE II

PRODUCTION OF ANOMERS FROM HYDROLYSIS OF XYLOTETRAOSE WITH HOMOGENEOUS XYLANASE AT 40° AND pH 5.5

Silylated anomer		*Time of elution (min)*	*Time of incubation (min)*				*Fully mutarotated standard*
			0.75	*6*	*20*	*120*	
			Percentage of species in anomeric form				
X_1	α	4.5	52.1	50.0	49.8	46.6	41.5
	β	5.0	47.9	50.0	50.2	53.4	58.5
X_2	α	12.3	36.9	31.0	19.1	17.8	17.1
	β	12.7	63.1	69.0	80.9	82.2	82.9
X_3	α	18.0	37.1	29.5	30.2	<20	<20
	β	18.3	62.9	70.5	69.8	>80	>80

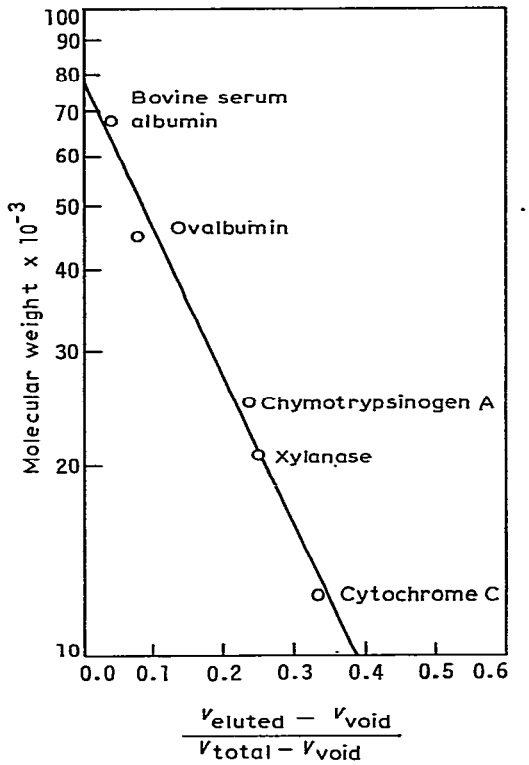


Fig. 7. Determination of molecular weight of xylanase by column chromatography on Sephadex G-50 Superfine.

286), pectin, and galacturonan, ~3, 10, and 23%, respectively, that of soluble xylan.

Samples of soluble Sigma xylan, the X_5–X_9 mixture, pectin, and galacturonan were incubated at 40° with homogeneous xylanase for 72 h, until hydrolysis approached completion. Based on a xylose standard, the enzyme liberated 11% as many reducing groups from pectin and 2.3% as many from galacturonan as from xylan, and 3.6 and 0.8% as many, respectively, as from the X_5–X_9 mixture.

Product configuration. — Products of enzymic hydrolysis of X_4 had a higher degree of α configuration than could be supplied by the reactant or by products initially present as contaminants (Table II). With increasing time X_1, X_2, and X_3 mutarotated to mixtures containing higher amounts of β anomer.

Molecular weight. — A molecular weight of 20,800 was measured by gel-permeation chromatography on Sephadex G-50 Superfine (Fig. 7).

Amino acid profile. — The amino acid profile of this homogeneous xylanase is shown in Table III, and is there compared with the profiles of homogeneous xylanases reported by others.

Carbohydrate content. — Four determinations of carbohydrate concentration based on a glucose standard yielded values of 33.7 ±9.6%. The range here, and in later text and graphs, gives the 95% confidence limits. The rest of the sample is

TABLE III

AMINO ACID COMPOSITIONS OF HOMOGENEOUS XYLANASES

Amino acid	Aspergillus niger[a]		Aspergillus niger[b]		Schizophyllum commune[c]		Stereum sanguinolentum[d]		Streptomyces sp.[e]
	Residues	*Mole %*	*Residues*	*Mole %*	*Residues*	*Mole %*	*Residues*	*Mole %*	*Mole %*
Aspartic acid	13.8	10.3	30	14.2	31.6	10.7	18.6	8.6	12.4
Threonine	9.3	7.0	32	15.1	29.7	10.1	26.8	12.3	7.8
Serine	12.2	9.2	28	13.2	37.5	12.7	34.1	15.7	8.2
Glutamic acid	14.1	10.6	17	8.0	19.0	6.4	15.1	6.9	8.1
Proline	8.2	6.2	5	2.4	13.7	4.6	11.4	5.2	4.2
Glycine	17.3	13.0	36	17.0	47.0	15.9	26.4	12.1	11.7
Alanine	12.8	9.6	13	6.1	20.9	7.1	17.9	8.2	9.4
Valine	6.8	5.1	1	0.5	14.4	4.9	13.2	6.1	5.4
Methionine	0.0	0.0	2	0.9	1.4	0.5	2.2	1.0	1.5
Isoleucine	6.7	5.0	5	2.4	11.7	4.0	6.6	3.0	4.0
Leucine	7.5	5.6	5	2.4	11.0	3.7	7.6	3.5	5.6
Tyrosine	4.0	3.0	16	7.5	23.0	7.8	10.7	4.9	3.9
Phenylalanine	3.6	2.7	5	2.4	5.0	1.7	6.8	3.1	4.1
Lysine	7.3	5.5	6	2.8	7.1	2.4	3.6	1.7	3.8
Histidine	3.7	2.8	2	0.9	4.1	1.4	2.4	1.1	2.2
Arginine	3.7	2.8	6	2.8	5.8	2.0	2.0	0.9	5.8
Cystine/2	2.1	1.6	Tr.[f]	Tr.	4.0	1.3	3.3	1.5	1.9
Tryptophan	—	—	3	1.4	8.5	2.9	8.6	4.0	—
Total residues	133		212		295		218		
Molecular weight	20,800		27,200		32,00		22,800		40,000

[a]This work. [b]See ref. 3. [c]See ref. 6. [d]See ref. 7. [e]See ref. 15. [f]Tr.; trace.

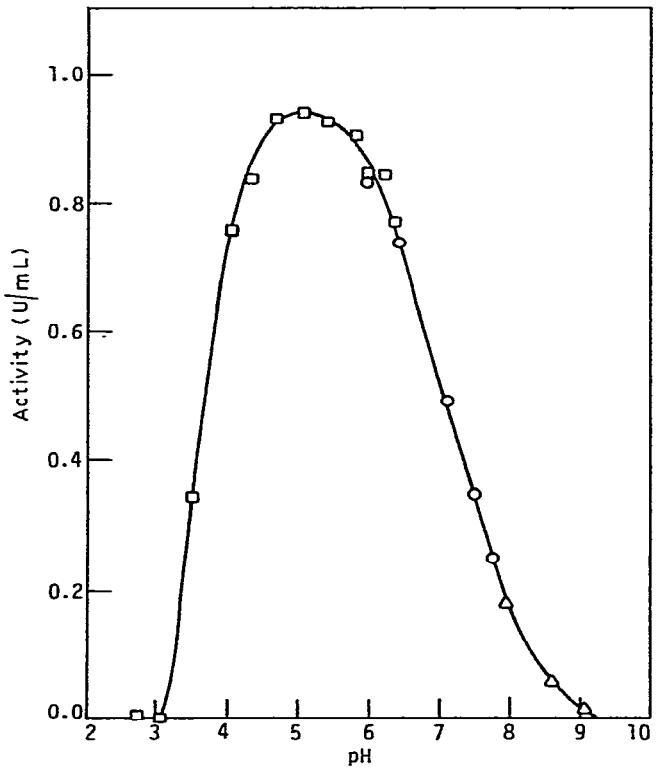


Fig. 8. Effect of pH on the activity of homogeneous xylanase at 40° in 0.035M sodium citrate (□), 0.035M sodium phosphate (○), or 0.035M sodium borate (△).

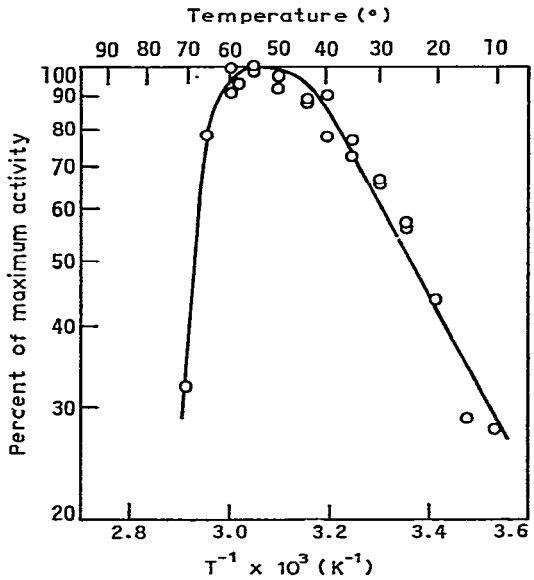


Fig. 9. Effect of temperature on the activity of homogeneous xylanase at pH 5.0.

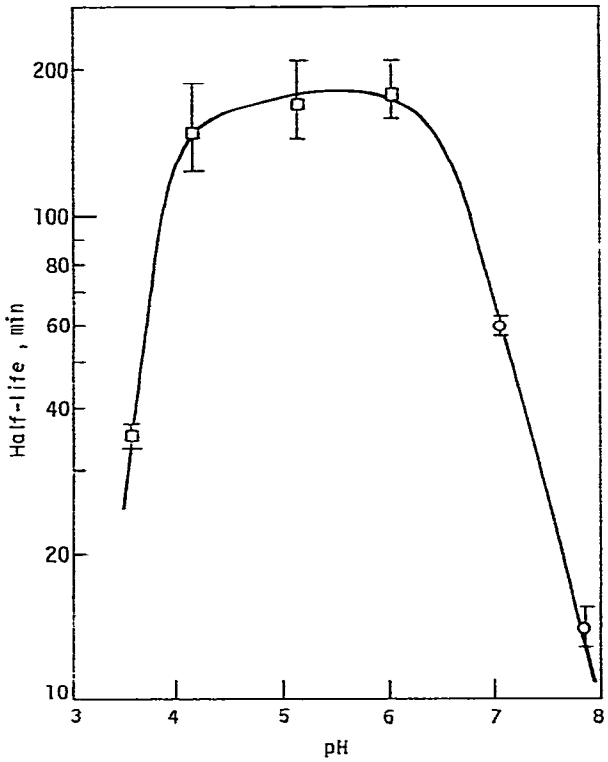


Fig. 10. Effect of pH on the stability of homogeneous xylanase in 0.035M sodium citrate (□) or 0.035M sodium phosphate (○).

assumed to be protein, measured with an ovalbumin standard. No hexosamines were detected by the Lee–Montgomery procedure[29].

Effect of pH and temperature on activity and stability. — Homogeneous xylanase was assayed in the standard manner, except at various pH values with various buffers. At all pH values, the ionic strength was adjusted to 0.5M with potassium chloride. Blanks without enzyme and calibrations with various amounts of xylose were made at each pH value. The highest activity was near pH 5 (Fig. 8).

The standard assay was conducted at various temperatures with samples of the homogeneous enzyme. The highest activity was found at 55°; up to 45° the activation energy was 26.2 ±4.6 kJ/mol (Fig. 9).

Decay of the enzyme was first-order throughout. Stability was highest between pH values of 4.5 and 6 (Fig. 10). Freezing (~—20°) of 0.1 mL of homogeneous enzyme with 0.75 mL of sodium acetate buffer of 0.75M at pH 5, either with or without 0.25 mL of 1% xylan, followed by thawing after 2 days and subsequent refreezing for another 2 days, led to no loss of activity. Evaporation to dryness at 40°, followed by reconstitution and by incubation for 2 h with xylan at 40°, caused ~25% loss of activity.

Effect of metal ions on activity. — Samples of 25 μL homogeneous xylanase

were assayed in the normal manner with various concentrations of a number of metal chlorides. The Hg^{2+} ion was inhibitory at 0.075mM and above, Co^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} at 7.5mM and above, and Al^{3+}, Ba^{2+}, Ca^{2+}, Cr^{2+}, and Ni^{+} at 75mM. The ions K^{+}, Li^{+}, and Na^{+} were not inhibitory at 75mM.

DISCUSSION

Xylanases that form large amounts of xylobiose and xylose as final products, but have not been shown to produce L-arabinose from xylan, have been previously reported from *Aspergillus*[30-33], *Schizophyllum*[5,6], *Stereum*[7], and *Streptomyces*[34]. In addition, *Trametes* can cleave xylotriose to xylose and xylobiose, but at such low rates that xylose hardly accumulates in the hydrolyzate[14,35].

The xylanase described in this paper is most similar to the *A. niger* enzyme of Sinner and Dietrichs[2], in that the molecular weight, optimal temperature and pH, and isoelectric point are essentially identical. However, no xylan containing L-arabinosyl side-groups were offered to their xylanase[32], and no amino acid profile was obtained by them, and so it is not possible to establish whether or not the two enzymes are identical.

Our xylanase may be the H-I described by Fukumoto *et al.*[1], which has a pI above 6, and a similar optimal pH (ref. 1) and action pattern[30,34]. However, it differs from that of Gorbacheva and Rodionova in amino acid profile, carbohydrate content, pI, molecular weight[3], and in the latter's production of transfer products and traces of L-arabinose from arabinoglucuronoxylan[33]. All six of the endo-xylanases from *A. niger* reported by John *et al.*[4] cleave L-arabinose from xylan, and, in addition, the molecular weights, as well as some optimal pH values and temperatures, differ from those of the xylanase we have described.

The xylanase reported here is closest in amino acid composition to one from *Streptomyces* (Table III), the only one of the five homogeneous xylanases of known amino acid profile that is not of fungal origin. It is most different from the other *A. niger* xylanase. Often its individual amino acid levels are either at the high or low end of the five. As with other xylanases and cellulases, its acidic amino acid content is high.

Although this enzyme is undoubtedly an endo-xylanase, as demonstrated by its extremely rapid production of xylo-oligosaccharides of medium length from xylan (Fig. 6), the configuration of the products is inverted, a property more commonly ascribed to exo-hydrolases[16]. As with other exo- and endo-hydrolases, but not glycoside glycohydrolases, such as β-D-xylosidase, this xylanase is more active on larger substrates, attack on xylotriose being very slow and that on xylobiose nonexistent.

Highly heterogeneous xylans were used in this project for a purpose: to allow the discovery of all enzymes present in the starting mixture that could cleave the various bonds in xylan. Despite this, the xylanase described here was highly specific in its action; it could not attack insoluble xylan at all, unless arabinosyl-initiated branches were removed (and then only slightly) but it was highly active on the more-

heterogeneous (and presumably more branched) soluble xylan. While it eventually hydrolyzed linear xylo-oligosaccharides to xylobiose and xylose, some fragments from hydrolysis of xylan were resistant to attack. This result indicated that the enzyme could not cleave bonds close to branch points or to residues other than xylose and, in fact, no arabinose or free uronic acid was ever produced from hydrolysis of xylan. What was left at the end of hydrolysis of a mixture of soluble and insoluble xylan, besides xylobiose and xylose, was the insoluble xylan, which was relatively high in xylose, and oligosaccharides from hydrolysis of soluble xylan, which conversely were high in arabinose and uronic acids.

On a molar basis, xylose was being produced at a higher rate towards the end of xylan hydrolysis than was xylobiose. This never occurred when the substrate consisted only of linear xylo-oligosaccharides, and it suggested that near branch points or non-xylosyl residues the action pattern of xylanase changed to favor endwise attack.

The attack of this xylanase on pectin and galacturonan requires further study. While the total number of reducing groups released is only $\sim 10\%$ of the number of xylosyl residues present in the pectin, and considerably less than that in the galacturonan, l.c. analysis of the products has not completely eliminated the possibility that bonds between uronic acid residues are being broken.

Such xylanases as the one described in this paper play a central role in the breakdown of xylan, producing small oligosaccharides for final hydrolysis by β-D-xylosidase. It is hoped that this study has further clarified their capabilities and limitations.

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