Xylanase from Trichoderma pseudokoningii: Purification, Characterization, and Effects on Isolated Plant Cell Walls

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ABSTRACT

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Trichoderma pseudokoningii produced an extracellular endo-β-1,4 xylanase when grown in shake culture at 25 C on a mineral salts medium containing 0.1% β-1,4 xylan as the sole carbon source. Enzyme activity was determined by measuring the release of reducing groups from xylan or *O*-hydroxyethyl xylan. Dialyzed culture filtrates from 4-day-old cultures were subjected to ion exchange chromatography on CM-Sephadex (C-25) in 20 mM sodium acetate buffer (pH 5.0); xylanase was eluted with a linear salt gradient (0-150 mM NaCl in buffer). Fractions containing the enzyme were dialyzed, lyophilized, dissolved in water, and subjected to gel filtration on Bio-Gel P-10 equilibrated with 50 mM sodium acetate buffer (pH 5.0) containing 100 mM NaCl. This two-step procedure yielded a 28-fold purification of the xylanase. The purified enzyme released only oligomers of xylose from β-1,4 xylan. It did not hydrolyze arabinan (araban), carboxymethylcellulose, β-1,4 galactan, mannan, glucomannan, sodium polypectate, or polygalacturonic acid. It had a pH optimum of 5.0, a pI of about 9.6, a molecular weight of 15,000 to 22,000, and was stable in 50 mM sodium acetate buffer (pH 5.0) at −20 C for up to 9 mo. The purified endo-β-1,4 xylanase readily solubilized carbohydrate containing xylose and arabinose from isolated cell walls of 5-day-old corn seedlings, and solubilized, to a lesser extent, carbohydrate containing primarily xylose from isolated cell walls from hypocotyls of 7-day-old bean seedlings. This enzyme should be valuable in evaluating the role of xylanase in cell wall hydrolysis by pathogens and in helping to further elucidate plant cell wall structure.

Additional key words: cell wall degradation, xylan.

The D-xylans are polysaccharides found in the hemicellulosic fraction of higher plant cell walls. These polymers consist of a β-1,4-linked D-xylopyranose chain which commonly has side branches of α-1,3-linked L-arabinofuranose and α-1,2-linked D-glucuronopyranose (or its 4-*O*-methyl ether). The number and kinds of branch residues are characteristic for different plant groups. Some xylans are acetylated at carbons 2 and 3 of the xylopyranose residues (35).

The D-xylans are found in almost all land plants (31) and they are the principal components of the primary cell walls of monocots (7). Since a number of microorganisms pathogenic to such plants are known to produce xylanases (5, 6, 10, 25, 30, 33), it is of interest to know whether or not the degradation of these polysaccharides plays a role in pathogenesis. The degradation of the xylopyranosyl chain by microorganisms can be accomplished through random hydrolytic cleavage of the β-1,4 glycosidic bonds by xylanases (8, 9, 11, 12, 17, 34). To study the effects of these enzymes on plant cell walls and to evaluate their possible role in pathogenesis, pure enzyme preparations are needed. Such preparations also would serve as powerful tools in the further elucidation of the plant cell wall structure. A survey of several microorganisms that produce xylanases that could be purified readily indicated that *Trichoderma pseudokoningii*, a nonpathogen, would be a good source for this enzyme. Here we report the purification and characterization of an endo-β-1,4 xylanase (EC 3.2.1.8) produced by *T. pseudokoningii* and present evidence that cell wall sugars are released from isolated corn (*Zea mays* L.) and bean (*Phaseolus vulgaris* L.) cell walls by this xylanase. Preliminary reports of these studies have been made (2, 4).

MATERIALS AND METHODS

Commercial xylan (larchwood) was obtained from ICN Pharmaceuticals, Inc. Cleveland, OH 44128. Cytochrome C from horse heart, horseradish peroxidase, ribonuclease A, and α-chymotrypsinogen A were obtained from Sigma Chemical Co., St. Louis, MO 63178. Ampholines for isoelectric focusing were purchased from LKB Instruments, Inc., Rockville, MD 20852. Carboxymethylcellulose (Type 7 MP) was obtained from Hercules Inc., Wilmington, DE 19899. Polygalacturonic acid was purchased from the Sunkist Growers, Inc., Ontario, CA 91764. Mannan was purchased from K & K Laboratories, Plainview, NY 11803, and araban was obtained from Koch-Light Laboratories, Ltd., Colnbrook, U.K. Beta-1,4 galactan was purified from *Lupinus albus* L. by the procedure of Jones and Tanaka (13). Glucomannan was prepared from the commercial xylan as described below. Xylan was further purified from the commercial larchwood xylan

(approximately 36% xylose) by adapting a method used by Meier (23) to purify hemicelluloses. Twenty-five g of the commercial xylan were dissolved in 150 ml of 10% NaOH. This solution was stirred while 20 ml of a saturated $Ba(OH)_2$ solution was added. The resulting precipitate was removed by centrifugation at 17,000 *g* for 15 min, and used later as a source of glucomannan. To the supernatant liquid, three additional amounts (20, 45, and 145 ml) of $Ba(OH)_2$ solution were added sequentially. After each addition, a precipitate formed which was removed by centrifugation. The first (glucomannan-containing) and final (xylan-containing) precipitates (fractions I & IV) were each suspended in 50 ml of distilled water. The two suspensions (fractions I & IV) along with the final supernatant liquid (fraction V), were each acidified with glacial acetic acid to approximately pH 5 and the resulting suspensions were dialyzed against water for 3 days at room temperature. The three fractions then were frozen and lyophilized (recovery: I, 6g; IV, 1.8 g; V, 2 g). The fractions were assayed for sugar content using the procedure of Jones and Albersheim (14) except that samples were hydrolyzed in 2 N trifluoroacetic acid for 90 min and no enzymatic treatment was used. Fraction I which contained 20% xylose was used as a crude source of glucomannan. Of the total recoverable sugar, fractions IV and V each contained about 90% xylose: IV, 91% xylose, 5% mannose, 4% glucose; V, 90% xylose, 10% glucose. Fraction IV was used as a xylan carbon source in culture media. Fraction V was used both as substrate for enzyme assays and for the preparation of *0*-hydroxyethyl xylan (OHEX) which was also used as an enzyme substrate.

A soluble derivative of xylan, OHEX, was prepared from xylan (fraction V) by a procedure similar to that used for the production of hydroxyethyl cellulose (18). Two grams of fraction V xylan were dissolved in 40 ml of 10% aqueous NaOH. This solution was cooled to 4 C and 5 ml of ethylene oxide was added. The flask containing the mixture was capped with aluminum foil and transferred to a water bath at 55 C and stirred for 2 hr. Prolonged heating of the alkaline solution was avoided in order to minimize polymer breakdown. The preparation was acidified with glacial acetic acid to pH 5, cooled, and dialyzed 48 hr against distilled water at 4 C. The final volume was brought to 200 ml (equivalent to about 1% xylan) and Thimerosal (0.005%) was added to retard microbial growth. The preparation was stored at 4 C.

Enzyme assays.—Xylanase was assayed by measuring the release of reducing groups from a substrate as determined by Nelson's procedure (27). Reaction

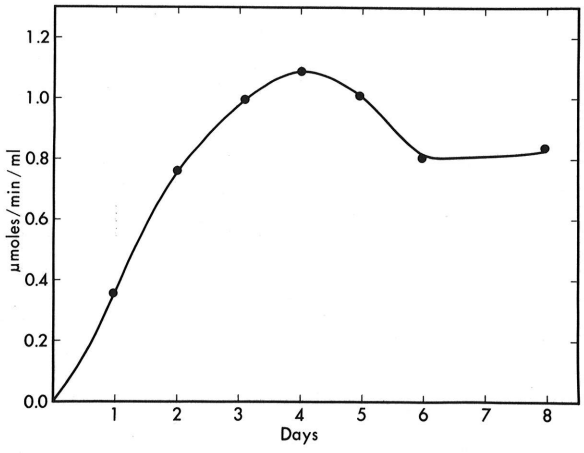

Fig. 1. Xylanase activity in culture filtrates of *Trichoderma pseudokoningii* during an 8-day growth period on a mineral salts medium containing 0.1% xylan. The enzyme was assayed at 30 C by measuring the release of reducing groups in reaction mixtures containing: 0.2 ml 1% *0*-hydroxyethyl xylan, 0.1 ml 200 mM sodium acetate buffer (pH 5.0), 0.05 ml dialyzed culture filtrate, and water to give a final volume of 0.5 ml.

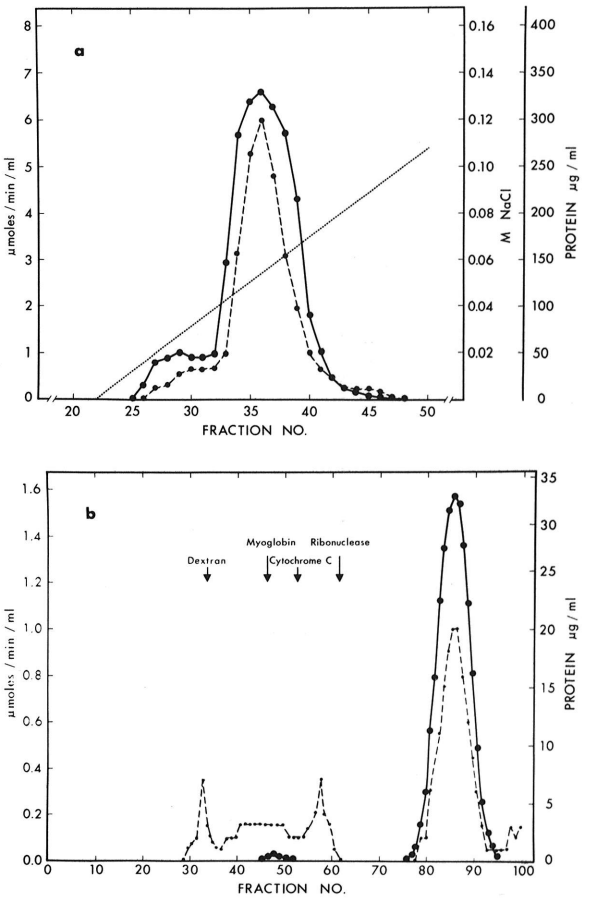

Fig. 2-(a, b). a) Elution profile from a CM-Sephadex (C-25) column of the xylanase components in culture filtrates of *Trichoderma pseudokoningii*. Crude culture filtrate was dialyzed against 20 mM sodium acetate buffer (pH 5.0) and applied to a CM-Sephadex column (1.5 cm × 21 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.0). The column was washed with buffer and then eluted with a NaCl gradient (····) in buffer. Fractions (7.5 ml) were collected and assayed as described in Fig. 1. Each fraction was assayed for protein (- - - -) and xylanase (——) activity. **b)** Elution profile of xylanase [pooled CM-Sephadex peak fractions (33-40)] from a Bio-Gel P-10 column (1.8 × 85 cm) equilibrated with 50 mM sodium acetate buffer (pH 5.0) and 100 mM NaCl. The markers indicate the elution peaks of: Blue dextran (mol. wt. 2,000,000), myoglobin (mol. wt. 17,500), ribonuclease (mol. wt. 13,700), and cytochrome C (mol. wt. 12,300). Enzyme activity (——) and protein content (- - - -) of each fraction were determined.

TABLE 1. Purification of endo-β-1,4 xylanase from culture filtrates of *Trichoderma pseudokoningii* grown for 4 days on a mineral salts medium containing 0.1% xylan

Fraction[a]	Volume (ml)	Total Protein (mg)	Specific[b] activity (μmoles/min/mg protein)	Recovery (%)
Culture filtrate	840	114	4.9	...
CM-Sephadex peak	85	7	58.7	74
Lyophilization	8	7	66.7	84
Bio-Gel (P-10) peak	50	2	137.1	49

[a]See methods section for preparation of the fractions.
[b]Enzyme activity was assayed at 30 C by measuring the increase in reducing groups in reaction mixtures containing: 0.2 ml 1% *O*-hydroxyethyl xylan, 0.1 ml 200 mM sodium acetate buffer (pH 5.0) and 0.2 ml enzyme solution.

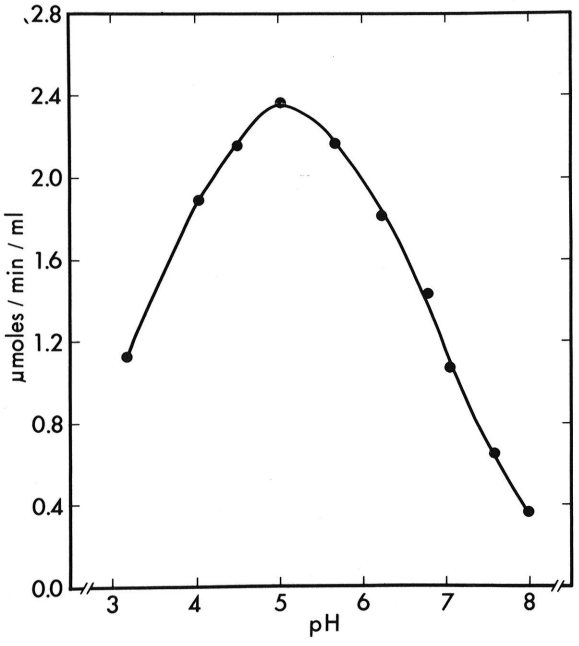

Fig. 3. Activity of the purified xylanase from *Trichoderma pseudokoningii* in relation to pH. Enzyme activity at 30 C was determined using 0.1 ml sodium citrate (pH 3), sodium acetate (pH 4-6), and sodium phosphate (pH 6-8) buffers (200 mM) in reaction mixtures containing 0.2 ml *O*-hydroxyethyl xylan (OHEX) and 0.1 ml aqueous solution of purified enzyme (10 ng).

mixtures contained: 0.2 ml 1% OHEX or 0.3 ml 0.5% xylan (fraction V), 0.1 ml 200 mM sodium acetate buffer (pH 5.0), 0.1 ml enzyme solution, and glass distilled water to make a final volume of 0.5 ml. The initial rate of substrate hydrolysis was proportional to the amount of enzyme present. Protein was determined by the procedure of Lowry et al. (20); bovine serum albumin was used as a standard.

Xylanase production, purification, and characterization.—The culture medium employed for enzyme production contained: 1.0 g NH_4NO_3, 650 mg KH_2PO_4, 181 mg $MgSO_4$, 149 mg KCl, 6.9 mg $MnSO_4 \cdot H_2O$, 3.5 mg $ZnSO_4 \cdot H_2O$, 3.3 mg $FeCl_3 \cdot 6H_2O$, 3.1 mg $CuSO_4 \cdot 5H_2O$, 1.0 g yeast extract (Difco), and 1.0 g xylan (fraction IV) in 1 liter of distilled water. This medium was stirred for 1 hr at 50 C to suspend the xylan; 100 ml amounts were dispensed into 500-ml flasks and autoclaved for 20 min [121 C, one atmosphere (15 psi)]. Stock cultures of *Trichoderma pseudokoningii* Rifai (isolate C-3) were maintained at 25 C on potato dextrose agar (PDA) slants. Mycelia on agar chunks from 2-day-old PDA slant cultures were used to seed the culture medium. Cultures were incubated at 25 C on a reciprocal shaker operated at 80 cycles/min.

Filtrates from 4-day-old cultures of *T. pseudokoningii* grown on the 0.1% xylan medium were filtered through four layers of cheesecloth. All subsequent steps were carried out at 4 C. The culture filtrates were centrifuged at 10,000 *g* for 25 min to remove debris and dialyzed 24 hr against 20 mM sodium acetate buffer (pH 5.0). This preparation was applied to an ion exchange column (1.5 cm × 21 cm) of Sephadex (C-25) equilibrated with 20 mM sodium acetate buffer (pH 5.0). It was critical to apply the preparation at pH 5.0 since at pH 5.5 the xylanase did not bind tightly. The column was washed with 150 ml of buffer and eluted with a linear salt gradient, 0-150 mM NaCl in 500 ml of buffer. The flow rate was 30 ml/hr and the eluent was collected in 10-ml fractions. Fractions were assayed for xylanase with OHEX as the substrate; the peak fractions were pooled, dialyzed against distilled water, and lyophilized.

The lyophilized enzyme preparation was redissolved in 8 ml of 50 mM sodium acetate buffer (pH 5.0) and 100 mM NaCl. Two-ml portions were applied to a column (1.8 × 90 cm) of Bio-Gel P-10 [74-38 μm (200-400 mesh)] equilibrated with the same salt-buffer mixture used to redissolve the enzyme. The column flow rate was adjusted to 6 ml/hr and the eluent was collected in 2 ml fractions. The peak enzyme fractions were pooled, dialyzed 18 hr against 50 mM sodium acetate buffer (pH 5.0), and stored at −20 C until used.

The molecular weight of the purified xylanase was estimated by sucrose density-gradient ultracentrifugation and by SDS discontinuous gel electrophoresis. The sedimentation behavior of the enzyme in a 5-20% sucrose gradient in 50 mM sodium acetate buffer (pH 4.5) was examined following the procedure of Mount et al. (24). Cytochrome C (mol. wt. 12,300) and horseradish peroxidase (mol. wt. 40,000) were used as standards. Cytochrome C was detected by the procedure of Lowry et al. (20) and peroxidase activity was measured by the method of Loebenstein and Linsey (19). The molecular weight of xylanase was estimated according to the

method of Martin and Ames (22). Sodium dodecyl sulfate (SDS) discontinuous gel electrophoresis was employed following the procedure of Maizel (21). Slab gels were 1.5 mm thick and 18-μliter samples were applied in 4.5 mm slots. The stacking gel and resolving gel were 3% and 13% polyacrylamide, respectively. An operating voltage of 50 V was applied until the tracking dye reached the resolving gel at which time the voltage was increased to 100 V. Alpha-chymotrypsinogen-A (mol. wt. 25,700), ribonuclease A (mol. wt. 13,700), and cytochrome C (mol. wt. 12,300) were used as standards.

The homogeneity of the xylanase preparation was checked by low-pH discontinuous gel electrophoresis at 4 C following the methods of Maizel (21). Gel dimensions and sample size were the same as described for the SDS gel electrophoresis. In order to determine the location of enzyme activity, some gels were not stained after electrophoresis and were cut into 5 mm segments. Each segment was crushed and eluted with 500 μliters of 200 mM sodium acetate buffer (pH 5.0) and assayed for enzyme activity.

The isoelectric point of the enzyme was determined by isoelectric focusing by the method of Bateman et al. (5). A rough estimate of the isoelectric point was made with pH 3-10 Ampholine carriers. A second electrofocusing was made with a (1:2, v/v) mixture of pH 8-10 and pH 9-11 Ampholine carriers. After 2 to 3 days the electric power supplied to the column stabilized at 0.37 W and fractions were collected. After determining the pH of the fractions, they were dialyzed overnight against water at 4 C and assayed for xylanase activity with OHEX.

Reaction products released by the purified xylanase from xylan were examined by paper chromatography. Reaction mixtures contained 10 μliters of the purified enzyme which released approximately 0.05 μmoles of products/min in a normal enzyme reaction mixture (0.5 ml). Aliquots (100 μliters) of reaction mixtures and

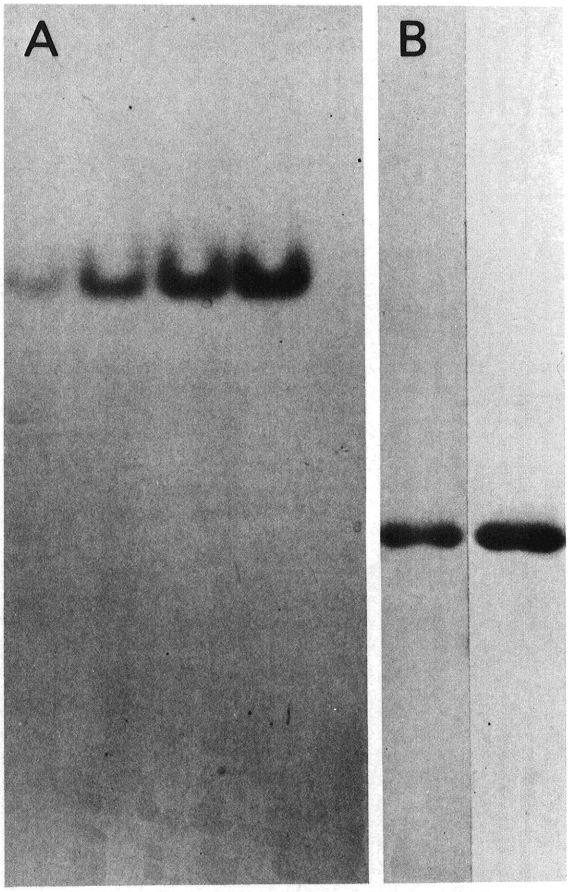

Fig. 4-(a, b). Slab gel electrophoresis of the purified xylanase from *Trichoderma pseudokoningii*. Gels for both SDS and low-pH electrophoresis were 1.5 mm thick and 18 μliter samples were applied in 4.5-mm-wide slots. The stacking gel (20 mm long) and resolving gel (90 mm long) were 3% and 13% polyacrylamide, respectively. An operating voltage of 50 V was applied until the tracking dye (bromphenol blue) reached the resolving gel at which time the voltage was increased to 100 V. Low-pH electrophoresis was run at 4 C and SDS electrophoresis at 25 C. **a)** Low-pH electrophoresis–samples from left to right contain: 3, 7.5, 12, and 15 μg, respectively, of purified xylanase. **b)** SDS electrophoresis–samples from left to right contain 6 and 15 μg of purified xylanase. Gels were stained with Coomassie Brilliant Blue R.

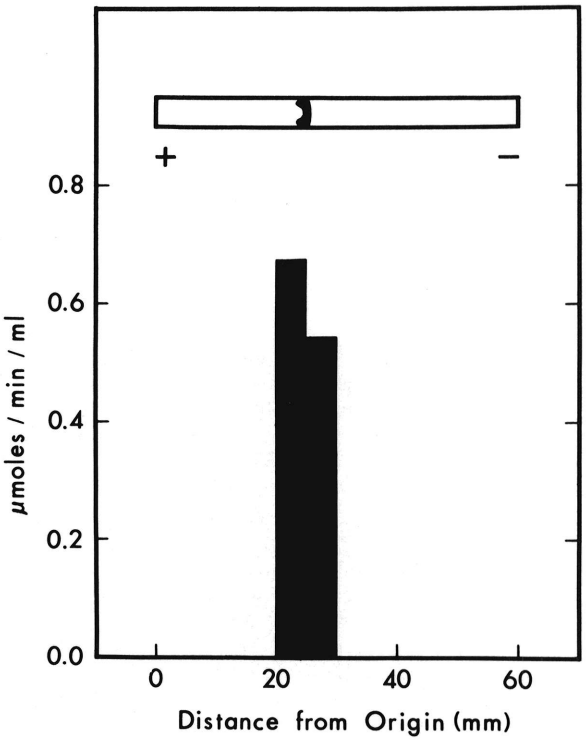

Fig. 5. Acrylamide gel electrophoresis (pH 5.0) of purified xylanase from *Trichoderma pseudokoningii*. Enzyme (15 μg) was applied to adjacent sample slots and subjected to electrophoresis at 4C. An operating voltage of 50 V was applied until the tracking dye (bromphenol blue) reached the resolving gel at which time the voltage was increased to 100 V. One section was stained for protein (top) and the other section was sliced into 5-mm segments; each segment was extracted with 0.5 ml 200 mM sodium acetate buffer (pH 5.0) and the extract was assayed for xylanase activity.

appropriate controls were spotted on chromatography paper after various intervals between 0 and 2 hr. The chromatograms were irrigated in a descending manner for 24 hr with a solvent mixture of butanol, acetic acid, and water (2:1:1, v/v), dried for 24 hr, and developed with silver nitrate reagent (32).

To check the specificity of the purified xylanase and to insure that it was not contaminated by other polysaccharidases, the activity of the purified xylanase on arabinan (araban), carboxymethylcellulose, β-1,4 galactan, glucomannan, mannan, sodium polypectate, and polygalacturonic acid was examined. Each reaction mixture contained 0.3 ml of a given polysaccharide (0.5%), 0.1 ml of a 500 mM buffer (sodium acetate, pH 4 or Tris-HCl, pH 8) and 0.1 ml enzyme or water. Thimerosal (0.005%) was added to prevent microbial contamination. Reaction mixtures were incubated 24 hr at 30 C and then assayed for increase in reducing groups by Nelson's method (27).

Cell wall studies.—Cell walls from hypocotyls of 7-day-old bean seedlings (*Phaseolus vulgaris* L.) and from 5-day-old corn shoots (*Zea mays* L.) were isolated by the method of Barnett (3). These preparations were further treated with organic solvents (6), air-dried overnight, and further dried over P_2O_5 for 48 hr under vacuum at 40 C. The release of cell wall constituents by the purified xylanase was determined in reaction mixtures containing: 10 mg cell walls, 1.5 ml enzyme (assayed activity on xylan, 4.6 μmoles/min/ml), 0.5 ml 200 mM sodium acetate buffer (pH 5.0), and Thimerosal (0.005%). Reaction mixtures were incubated for 18 hr at 25 C with constant stirring. Cell wall residues and supernatants of reaction mixtures were assayed for noncellulosic carbohydrate constituents by the procedure of Jones and Albersheim (14).

RESULTS

Production, purification, and properties of T. pseudokoningii xylanase.—A time-course study of xylanase production in culture, based on assays of dialyzed culture fluid, revealed that maximum activity occurred on day 4 (Fig. 1). When culture filtrate was applied to a CM-Sephadex column, the enzyme was eluted when the NaCl gradient reached 40 mM (Fig. 2-a). The majority of the culture filtrate protein (approximately 90%) did not bind to the column and was found in the void volume and buffer wash. These fractions did not contain significant xylanase activity. A minor peak of xylanase activity was eluted when the NaCl gradient reached 20 mM, but this peak was discarded. Lyophilization of pooled peak fractions seemed to enhance xylanase specific activity (Table 1). Xylanase eluted from the Bio-Gel P-10 column with an elution volume to void volume (V_e/V_o) value of 2.7 (Fig. 2-b). This V_e/V_o value is unusually high for an enzyme and suggests that factors other than gel filtration were involved in the elution of the enzyme.

The purified enzyme (Bio-Gel P-10 peak) was stored for up to 9 mo in 50 mM sodium acetate buffer (pH 5.0) containing 100 mM NaCl at −20 C without significant loss of activity.

The optimum pH for substrate hydrolysis by the purified xylanase was determined with sodium citrate (pH 3), sodium acetate (pH 4-6), and sodium phosphate (pH 6-8) buffers (200 mM) in the routine xylanase assay (Fig. 3). The pH optimum was about pH 5.0 with either OHEX or xylan as the substrate. The isoelectric point of the purified xylanase was about 9.6 as determined by column electrofocusing with Ampholine carriers.

The purified xylanase was shown to be homogeneous by electrophoresis. Low-pH discontinuous gel electrophoresis revealed a single protein band when as much as 15 μg of enzyme preparation was applied (Fig. 4-a); this band coincided with the location of xylanase activity eluted from nonstained gels (Fig. 5). Stained gels from SDS discontinuous electrophoresis also showed a single protein band (Fig. 4-b). The molecular weight of the enzyme was estimated by SDS electrophoresis to be about 15,000 daltons as determined by comigration of protein standards (Fig. 6). The molecular weight estimated by the method of Martin and Ames (22) was 20,375 daltons and 22,280 daltons, respectively, when cytochrome C (12,300 daltons) and horseradish peroxidase (40,000 daltons) were used as the reference proteins.

Paper chromatography of aliquots of reaction mixtures containing xylan and purified xylanase after 0, 30, 60, and 120 min of incubation at 30 C revealed that this enzyme released at least four oligomers, but no monomer (Fig. 7). Approximately 14% of the glycosyl bonds present in the substrate were hydrolyzed after 120 min of incubation. The oligomers were each eluted from the chromatogram with water, and then further degraded by a crude *T. pseudokoningii* enzyme preparation. Paper chromatography of the degraded oligomers showed that each contained only xylose. A quantitative assay of the reducing groups, before and after degradation of the

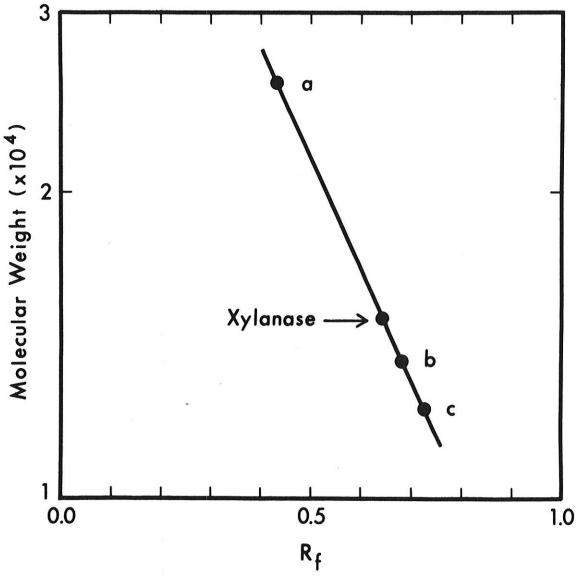

Fig. 6. Molecular weight estimate of the purified xylanase from *Trichoderma pseudokoningii* by SDS gel electrophoresis (See Fig. 4 for procedures). Reference proteins included: (a) α-chymotrypsinogen-A (mol. wt. 25,700), (b) ribonuclease (mol. wt. 13,700), (c) cytochrome C (mol. wt. 12,300). The estimated molecular weight of xylanase was approximately 15,000 daltons.

oligomers, suggests they are di-, tri-, tetra-, and pentamers.

When arabinan (araban), carboxymethylcellulose, β-1,4 galactan, glucomannan, mannan, sodium polypectate, and polygalacturonic acid were used as potential substrates for the purified xylanase, no release of reducing groups was detected, except in reaction mixtures containing glucomannan (Table 2). Upon analysis by paper chromatography of the products released from glucomannan, only xylose oligomers were detected. Gas chromatographic analysis (1) of the glucomannan substrate showed that it contained 15-20% xylose.

Release of carbohydrate from isolated bean and corn cell walls by purified xylanase.—Analysis of isolated bean and corn cell walls by the procedure of Jones and Albersheim (14) indicated that 59% and 39% of the wall material, respectively, was accounted for as specific sugars of noncellulosic carbohydrates (Fig. 8-a, b). The major sugars in the bean cell walls were galactose and galacturonic acid but those in the corn cell walls were xylose and arabinose. Analysis of the supernatant portions of reaction mixtures in which the purified xylanase was incubated with bean and corn cell walls showed that this enzyme solubilized carbohydrate from both of those sources. This enzyme solubilized only about 0.6% of the bean cell walls and the major sugar present in the carbohydrate released was xylose (Fig. 9-b). Xylanase released 4.5% of the corn cell wall as carbohydrate. The major sugars in this fraction were xylose and arabinose (Fig. 9-a). Analysis of the cell wall residues following treatment with xylanase showed that the xylose and arabinose contents of the corn cell walls and the xylose content of bean cell walls had been decreased by the enzyme treatment (Fig. 9-c, d). This analysis of corn cell wall residues also indicated that following xylanase treatment there was a slight increase in the amount of glucose and galacturonic acid.

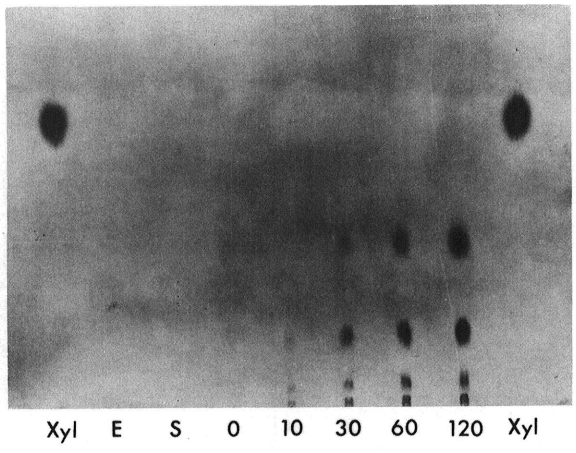

Fig. 7. Paper chromatogram of reaction products released from xylan by the purified xylanase from *Trichoderma pseudokoningii*. Reaction mixtures contained: 0.3 ml 0.5% xylan, 0.1 ml 200 mM sodium acetate buffer (pH 5.0), and 0.1 ml enzyme solution (activity = 0.05 μmoles of reducing groups released/min). One hundred μliter aliquots of reaction mixtures were spotted on chromatography paper at the indicated time intervals (min). Controls were enzyme (E), substrate (S) and 50 μg xylose (X). The chromatogram was irrigated in a descending manner for 24 hr with butanol, acetic acid, and water (2:1:1, v/v), dried for 24 hr and developed with silver nitrate reagent.

DISCUSSION

The endo-β-1,4 xylanase produced by *T. pseudokoningii* was purified by a relatively simple two-step scheme involving cationic ion exchange chromatography and gel filtration (Table 1). Purification of this enzyme was facilitated by its high isoelectric point (pI 9.6) and its low molecular weight (15,000 to 22,000 daltons). Another factor that greatly aided in the purification of the enzyme was its behavior during gel filtration. The V_e/V_o value of 2.7 exhibited by the xylanase following gel filtration in Bio-Gel P-10 (Fig. 2-b) is characteristic of compounds in the molecular weight range of 6,000. Thus, the V_e/V_o of this xylanase was unusually high when one considers its estimated

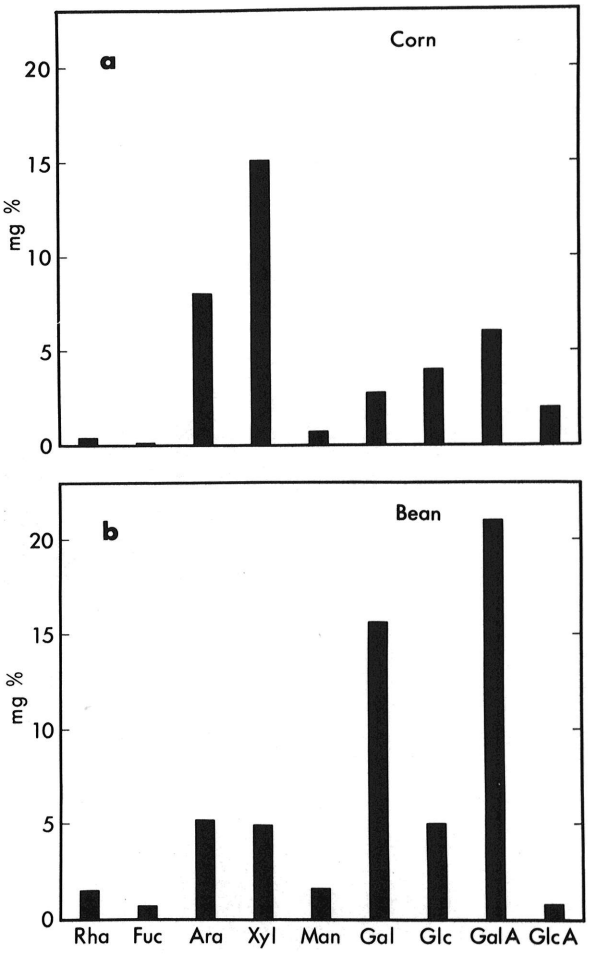

Fig. 8-(a, b). Neutral sugar and uronic acid composition (mg %) of **a)** 5-day-old corn shoots and **b)** 7-day-old bean hypocotyl isolated cell walls. Abbreviations: Rha = rhamnose; Fuc = fucose; Ara = arabinose; Xyl = xylose; Man = mannose; Gal = galactose; Glc = glucose; Gal A = galacturonic acid; and Glc A = glucuronic acid.

molecular weight as determined by SDS gel electrophoresis (Fig. 6) or sucrose density gradient ultracentrifugation. Lyophilization of the major xylanase peak eluted from the CM-Sephadex column resulted in a consistent increase in specific activity of this enzyme (Table 1). The reason for this effect is unknown.

Gel electrophoresis at low pH and SDS gel electrophoresis of the purified xylanase revealed that only one protein band was present. Furthermore, assays of unstained low-pH gels showed that xylanase activity and the protein band were coincident. This enzyme did not exhibit activity on any nonxylose containing polysaccharides (Table 2), demonstrating that it was specific for xylan and free of other polysaccharidases. Unlike a highly purified xylanase from *Stereum sanguinolentum* (8) which exhibited some activity on carboxymethylcellulose due to a trace amount of cellulase, the purified enzyme from *T. pseudokoningii* did not hydrolyze this substrate. The apparent activity of the purified *T. pseudokoningii* xylanase on glucomannan involved only the release of xylose oligomers from a preparation which contained about 20% polymeric xylose. Since the glucomannan was prepared from a commercial xylan preparation, the contamination of this substrate with xylan is understandable.

Like the xylanases reported for several fungi (9, 12, 17, 34), the purified xylanase of *T. pseudokoningii* has an acid pH optimum (pH 5.0) and cleaves xylan releasing oligomeric xylose, the smallest being a dimer. The absence of monomeric xylose in reaction mixtures indicates that this enzyme is unable to cleave xylose oligomers containing less than four xylose residues. A similar pattern of substrate specificity has been demonstrated for an endoxylanase from *Aspergillus foetidus* (34). Thus, the purified xylanase from *T. pseudokoningii* may be regarded as an endo-β-1,4 xylanase.

Our analysis of the carbohydrate constituents of cell walls of corn and bean seedlings (Fig. 8-a, b) by the procedure of Jones and Albersheim (14) yielded results comparable to those reported by others (1, 28). The purified xylanase from *T. pseudokoningii* released

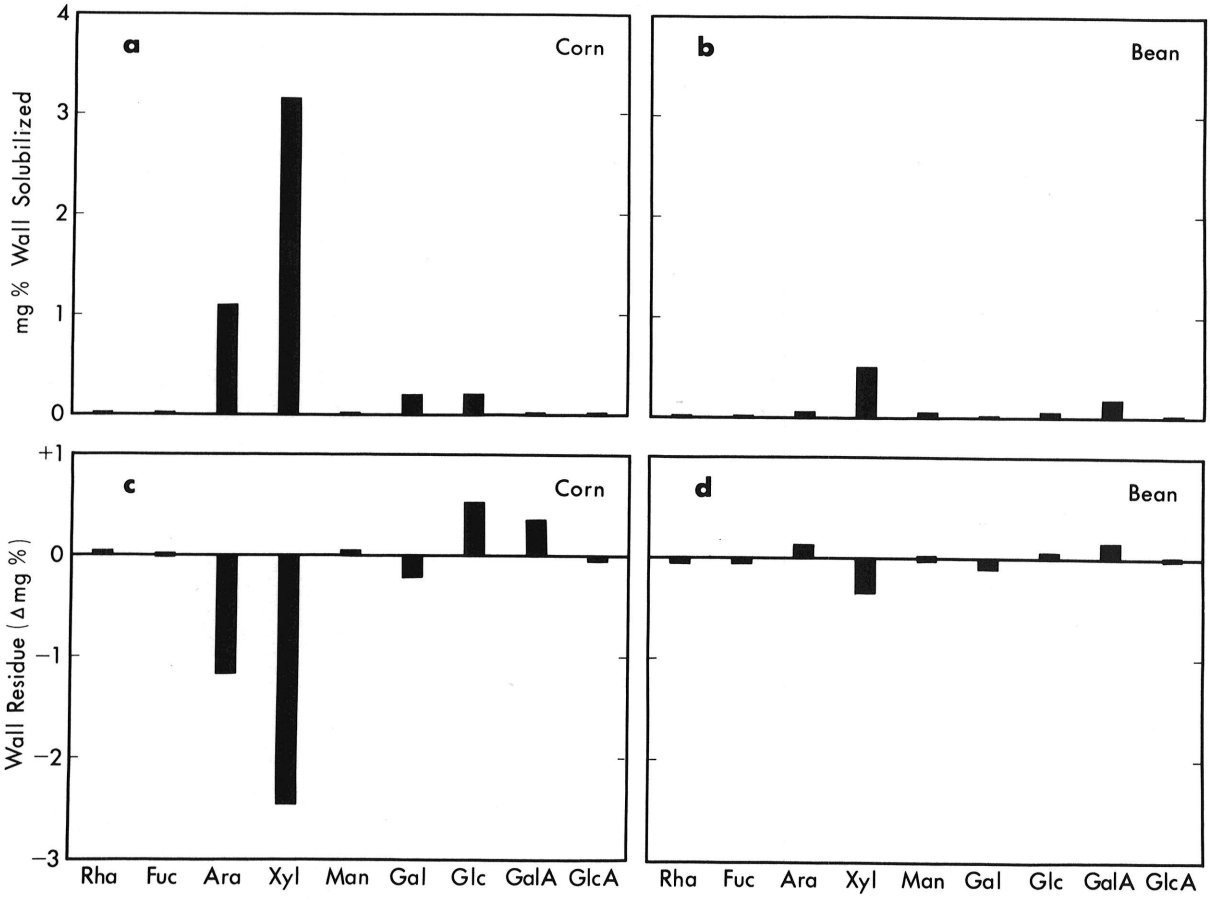

Fig. 9-(a to d). Solubilization of plant cell wall sugars by a purified xylanase from *Trichoderma pseudokoningii*. Reaction mixtures contained: 10 mg cell walls, 1.5 ml enzyme (assayed activity, 4.6 μmoles/min/ml), 0.5 ml 200 mM sodium acetate buffer (pH 5.0) and Thimerosal (0.005%). The reaction mixtures were incubated for 18 hr at 25 C with constant stirring. After enzyme treatment, cell walls and supernatants of reaction mixtures were assayed for constituent sugars. Results are compared to controls of cell walls in buffer and are expressed in mg %. **a, b)** Carbohydrates found in supernatants of xylanase-treated corn and bean cell walls, respectively. **c, d)** Changes in constituents of corn and bean cell wall residues, respectively, after treatment with xylanase.

TABLE 2. Tests for breakdown of selected polysaccharides by purified xylanase from *Trichoderma pseudokoningii*[a]

Polysaccharide	Reducing groups released (μmoles/24 hr) in reaction mixtures at pH: 4.0	8.0
Arabinan (araban)	0.00	0.00
Carboxymethylcellulose	0.00	0.00
β-1,4 Galactan	0.00	0.00
Glucomannan[b]	0.32	0.15
Mannan	0.00	0.00
Polygalacturonic acid	0.00	0.00
Sodium polypectate	0.00	0.00
Xylan	570.00[c]	107.00[c]

[a]Reaction mixtures contained 0.3 ml of a given polysaccharide (0.5%), 0.1 ml of a 500 mM buffer (sodium acetate, pH 4 or Tris-HCl, pH 8) and 0.1 ml enzyme. Reaction mixtures were incubated 24 hr at 30 C and then assayed for increase in reducing groups.

[b]Reaction products were demonstrated by paper chromatography to be oligomers of xylose.

[c]Calculated activity based on assays of reaction mixtures incubated for 10 min.

carbohydrate containing primarily xylose and arabinose from corn cell walls (Fig. 9-a). Since the xylan in monocots exists as arabinoxylan (35), it may be assumed that the arabinose solubilized was covalently linked to xylose. Only a small amount of carbohydrate was released from the bean cell walls by this xylanase, although the major constituent was xylose (Fig. 9-b). This was not an unexpected result since young bean cell walls with a relatively low xylose content were used in these experiments, and it is known that the xylose in the primary bean cell wall occurs as a xyloglucan (36), which should be resistant to hydrolysis by a purified xylanase. It is likely that the xylose released from bean cell walls represents the action of xylanase on β-1,4 linked xylan deposited during secondary wall thickening (29). Purified xylanase is capable of attacking its substrate within isolated plant cell walls without the prior action on the walls of a "wall modifying enzyme" (15) or polygalacturonase (16).

Cell walls of both monocots and dicots contain substantial amounts of β-1,4 linked xylans (35) and a number of plant pathogens produce enzymes that split the β-1,4 xylosyl bond in these cell wall polymers (5, 6, 10, 25, 30, 33). The presence of xylanase in diseased tissues has been demonstrated in several host-parasite systems (10, 26, 30, 33) and in some instances the xylose content of cell walls in lesions of diseased plants was reduced greatly (25, 33). Although xylanase has been demonstrated in sunflower tissues infected with *Sclerotinia sclerotiorum*, analysis of the xylose content of cell walls from such tissues failed to reveal a depletion in their xylose content (10). The role of xylanase in pathogenesis is unresolved, but in view of the large amounts of β-1,4 linked xylans in the cell walls of higher plants (7, 35) and the apparent ability of pathogens to produce enzymes which hydrolyze these polymers, the significance of these enzymes in disease and plant tissue breakdown needs to be resolved. The availability of a purified xylanase should facilitate further studies on the role of xylans in plant cell wall structure as well as studies on the role of these enzymes in cell wall breakdown by xylanolytic plant pathogens.

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