APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1978, p. 802-808
0099-2240/78/0036-0802$02.00/0

Vol. 36, No. 6
Printed in U.S.A.

Production, Characterization, and Partial Amino Acid Sequence of Xylanase A from *Schizophyllum commune*

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Received for publication 28 September 1978

Xylanase A, one of several extracellular xylanases produced by *Schizophyllum commune* strain Delmar when grown in submerged culture with spruce sawdust as carbon source, was purified 43-fold in 25% yield with respect to total xylanase activity. Although some polysaccharide was strongly bound to the purified enzyme, the complex could be dissociated by sodium dodecyl sulfate and appeared homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weight of the protein, calculated from the electrophoretic mobility, was 33,000. The molecular activity of the purified xylanase A, determined with soluble larch xylan as substrate, was 1.4×10^5 min^{-1}, with xylobiose and xylose as the major products. The enzyme had a pH optimum of 5.0 and a temperature optimum of 55°C in 10-min assays. The acid hydrolysate of xylanase A was rich in aspartic acid and aromatic amino acids. The sequence of 27 residues at the amino terminus showed no homology with known sequences of other proteins.

Xylans, or polymers of D-xylose, are widely distributed in plant cell walls. The anhydro-D-xylose units are generally linked $\beta(1 \rightarrow 4)$, and characteristic variations in structure (such as substitution by arabinose or uronic acid residues and acetyl groups) are found in different plant species (30). There is commercial interest in the production of xylose, and hence xylitol, by the acid hydrolysis of xylan-containing materials (22). Microorganisms can also hydrolyze xylan, and many xylanases [$\beta(1 \rightarrow 4)$-D-xylan xylanohydrolase (EC 3.2.1.8)] have been isolated and characterized (for a review, see reference 4). The hydrolysis of xylan by an immobilized xylanase has been investigated (28).

Because little is known about the structure or mechanism of action of xylanase enzymes, we undertook to produce purified enzymes suitable for structural studies and, ultimately, for the elucidation of the mechanism of their action.

Previously conducted screening experiments (15, 16) had indicated that *Schizophyllum commune*, a wood-destroying basidiomycete, was a potent producer of cellulase and xylanase in both stationary and submerged cultures. Early attempts to produce the enzymes were based on shake flask cultivation, with beech sawdust as the major carbon source (16). Small amounts of a xylanase have been isolated by electrophoresis (35). The present paper documents the production in a fermentor of a xylanase from *S. commune*, its isolation, characterization, and partial amino acid sequence.

MATERIALS AND METHODS

Microorganism. *S. commune* strain Delmar, selected by screening 16 strains (26), was maintained at 4°C on a malt agar slant.

Xylanase production. A 14-liter fermentor (Microferm; New Brunswick Scientific Co.), containing 400 g of black spruce sawdust (passing through a 40-mesh screen), 25 g of peptone (Difco Laboratories), 72 g of $Ca(NO_3)_2 \cdot 4H_2O$, 5 g of KH_2PO_4, 5 g of $MgSO_4 \cdot 7H_2O$, and 10 liters of tap water, was inoculated with a 3-day culture of the microorganism grown in 200 ml of vigorously stirred 1.5% malt extract broth (Difco). The initial pH in the fermentor was 3.9. The fermentor was operated for 9 days at 30°C, 200-rpm agitation, and 1.2-liter per min aeration rate. The culture solution was clarified by filtration through a Büchner funnel (without filter paper), followed by centrifugation (7,000 × *g* at 5°C for 10 min).

Xylanase A purification. The enzyme complex was precipitated by adding 3 volumes of ethanol to 1 volume of culture filtrate at −18°C. The precipitate was dissolved in 100 mM pyridine-acetic acid buffer (pH 5) and freeze-dried, yielding approximately 1 g of crude enzyme complex per liter of culture filtrate. Crude enzyme complex (4 g) was dissolved in 50 ml of 300 mM acetate-50 mM *N*-ethylmorpholine-ammonia buffer (pH 9) and applied to a diethylaminoethyl (DEAE)-Sephadex A-50 column (5 by 85 cm) equilibrated with the same buffer. The column was eluted with a linear gradient consisting of 4 liters of the above buffer and 4 liters of 100 mM pyridine-50 mM *N*-ethylmorpholine-acetic acid buffer (pH 5). Xylanase A, the first of several xylanases to be eluted, emerged at approximately one-third of the elution profile. Most of the other components of the complex, including brown pigments, remained adsorbed on the column.

Fractions containing xylanase A were partially freeze-dried and then passed through a Sephadex G-50 column (5 by 85 cm) equilibrated with 200 mM pyridine–acetic acid buffer (pH 5). The xylanase A peak, which appeared at two-thirds of the column bed volume, was collected and freeze-dried to yield 40 mg of homogeneous material.

Electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gels were prepared by the method of Weber and Osborn (37). The 10% gels (acrylamide/methylenebisacrylamide ratio, 39:1) were set in slabs (20 by 7 cm). The protein-SDS complex was obtained by heating the protein (1 to 2 mg) at 100°C for 5 min in a 1% SDS solution (1 ml) containing 6 M urea and 1% 2-mercaptoethanol at pH 7.0.

Xylan. Xylan from larchwood (molecular weight, 20,000; Sigma Chemical Co.) is an unbranched chain of xylopyranose residues, with every fifth or sixth residue substituted at C2 with a 4-*O*-methyl-D-glucuronic acid unit and with a small number of xylopyranose residues substituted at C3 with arabofuranose units (1). It is partially soluble in water; the water-soluble portion represents about one-third of the total weight at 30°C.

Xylanase and carboxymethylcellulase assays. Xylanase activity was routinely measured by incubating 1 ml of an appropriately diluted enzyme solution with 1 ml of a 1% larch xylan suspension in 200 mM sodium acetate buffer (pH 5) for 10 min at 30°C. For pH and temperature optima and kinetic experiments, xylan suspensions were centrifuged, and the resulting clear solutions were used as substrate. Reducing sugar concentration was determined by heating the solution at 100°C for 15 min with dinitrosalicylic acid reagent (21), diluting it with 1% aqueous Rochelle salt to 20 ml, and then reading the optical density at 575 nm. The optical density was calibrated with known concentrations of glucose solution. Appropriate blanks were used. Carboxymethylcellulase activity was determined by the same procedure, except that 1% carboxymethylcellulose (type 7 LT; Hercules Inc.) was used as substrate. Enzyme units were defined as the release of 1 μmol of reducing sugar (as xylose or glucose equivalents) per min. Protein concentrations were determined by the method of Lowry et al. (20). Crystalline bovine serum albumin (Schwarz/Mann) was used as standard.

Sugar analyses. Hydrolysis products were analyzed qualitatively by paper chromatography and quantitatively by gas chromatography. Paper chromatograms were developed with ethyl acetate–pyridine–water (8:2:1), and the components were detected with 3% *p*-anisidine hydrochloride (12). Gas chromatographic separation of trimethylsilyl ether derivatives was performed on a column (1.5 m by 0.3 cm) containing 3% OV-17 on 100/120-mesh Chromosorb W (Chromatographic Specialties Ltd.). The temperature program was 100°C → 200°C at 4°C/min and then 200°C → 300°C at 10°C/min. Approximate retention times (in minutes) were as follows: xylose, 12.4 and 13.8 (two anomers); xylobiose, 30.8; and xylotriose, 40.1. Peaks were quantitated by means of xylose response factors.

Amino acid analysis. Protein samples were hydrolyzed by the method of Moore and Stein (24). Hydrolysis times of 24, 48, and 72 h were used. The protein hydrolysates were analyzed on a Durrum amino acid analyzer. The closest-to-integral numbers of amino acid residues per molecule were calculated by using the molecular weight determined by electrophoresis as a guide. Serine and threonine values were obtained by linear extrapolation to zero-time hydrolysis. Since neither valine nor isoleucine values showed any significant increase with hydrolysis time, no special procedure was used to calculate them. Half-cystine residues were determined as cysteic acid after performic acid oxidation (23). Tryptophan residues were determined after hydrolysis with *p*-toluenesulfonic acid (19).

Amino acid sequencing. Automatic sequence analysis was performed with a Beckman sequencer model 890B. Residues were identified with a Durrum model D-500 amino acid analyzer after hydrolysis of portions of the anilinothiazolinone derivatives with 57% hydroiodic acid or, in the case of tryptophan, with 0.1 M NaOH–0.05 M $Na_2S_2O_4$ (32). These assignments were confirmed and augmented by conversion of the remainder of the thiazolinones to the phenylthiohydantoin derivatives by incubation with 0.2 ml of 1 M HCl at 80°C for 10 min (7) and subsequent identification of the derivatives by gas-liquid chromatography in a Beckman GC45 gas chromatograph (27) and by one-dimensional thin-layer chromatography on silica gel plates (6).

RESULTS

Xylanase production. The composition of the medium, temperature, and aeration conditions in the fermentor were deduced from previous studies (15–17) with shake flask cultures of *S. commune*. Preliminary experiments with the fermentor suggested that a combination of low stirring rate and limited aeration gave the best enzyme yield. Substitution of spruce for the previously used beech sawdust gave slightly lower enzyme activity, but also produced lesser amounts of pigmented impurities in culture filtrates and resulted in simpler enzyme purification.

Xylanase A purification. Precipitation of the culture filtrate with ethanol resulted in approximately 72% xylanase recovery. The precipitate could be stored in a freezer for at least 2 months with no substantial loss of activity. Precipitation with acetone resulted in extensive denaturation; ammonium sulfate precipitation was inconvenient due to the subsequent necessity of extensive dialysis. Ion-exchange chromatography of the ethanol precipitate on DEAE–Sephadex A-50 separated xylanase A from other xylanases and carboxymethylcellulases in the *S. commune* enzyme complex (26). The enzyme was further purified by Sephadex G-50 chromatography. Freeze-dried material from this column (yield, 10 mg/liter of the culture filtrate) showed only one protein band when subjected

to SDS-polyacrylamide gel electrophoresis (Fig. 1).

The purification procedure is summarized in Table 1.

Temperature and pH optima. The temperature optimum of xylanase A in a 10-min assay with soluble xylan was between 45 and 50°C (Fig. 2).

The enzyme exhibited a broad pH optimum around pH 5 (Fig. 3) and was stable within a pH range of from 6 to at least 8, as shown by the activity remaining after 65 h at 30°C (Fig. 4).

Hydrolysis of xylan with xylanase A. The products of incubation of purified xylanase A with larch xylan were analyzed by gas chromatography. After an 18-h hydrolysis, the major products were xylose (25%) and xylobiose (75%), with traces of xylotriose. Paper chromatography qualitatively confirmed this analysis. Xylan from esparto grass, which is a simple xylopyranose chain with no substituents and one

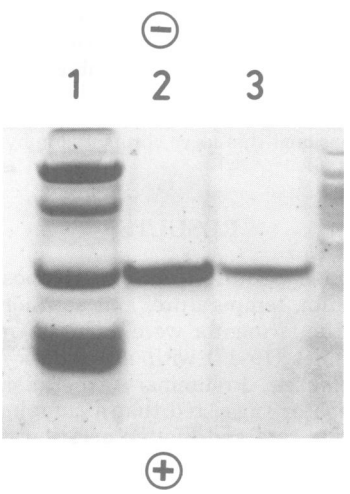

FIG. 1. *SDS-polyacrylamide gel electropherogram of xylanase A. (1) Protein standards (from the top): bovine serum albumin, ovalbumin, chymotrypsinogen A, myoglobin, and cytochrome c (last two merged). (2 and 3) Xylanase A at different loadings.*

TABLE 1. *Purification of xylanase A from S. commune*

Step	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act	Purification	Yield (%)
Crude culture filtrate	6,500	6,714	2,379	0.354	1	100
Ethanol precipitate	400	1,481	1,720	1.161	3.28	72.3
Eluted from DEAE-Sephadex	528	69.2	970	14.02	39.60	40.8
Eluted from Sephadex G-50	190	39.2	601	15.33	43.30	25.3

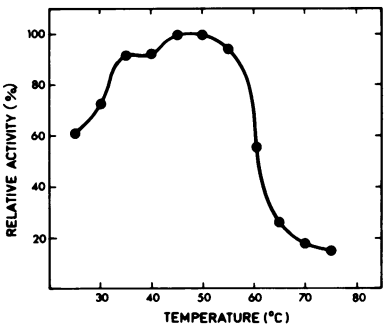

FIG. 2. *Effect of temperature on xylanase A activity. The assay mixtures were buffered with 0.2 M acetate at pH 5 and incubated for 10 min at each temperature with a 0.28% solution of larch xylan.*

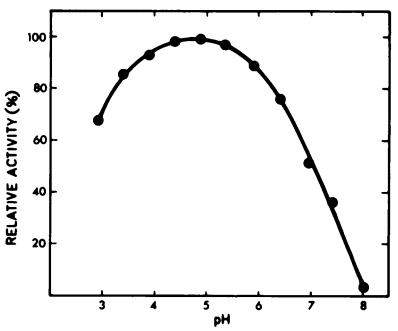

FIG. 3. *Effect of pH on xylanase A activity. The assay mixtures were buffered with 0.1 M citrate or 0.1 M phosphate and incubated for 10 min at 30°C with a 0.28% solution of larch xylan.*

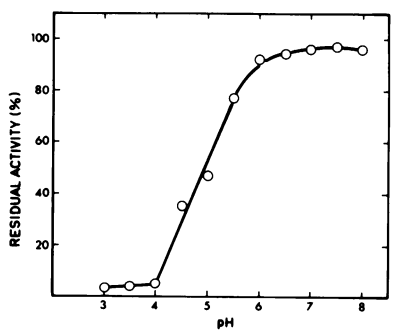

FIG. 4. *Effect of pH on xylanase A inactivation. The enzyme solutions were buffered with 0.1 M citrate or 0.1 M phosphate and left at 30°C for 65 h. The enzyme solutions were then diluted (and the pH was adjusted) with 0.2 M acetate at pH 5 and incubated for 10 min with a 1% suspension of larch xylan.*

C2 branch (2), was also hydrolyzed by the purified xylanase. Carboxymethylcellulose was not hydrolyzed at 100 times the enzyme concentration normally used in the standard xylanase assay.

Kinetics. With a clear solution of larch xylan as substrate, the Eadie-Hofstee plot (Fig. 5) gave a K_m of 8.37 mg/ml and a V_{max} of 0.443 μmol/min. The amount of enzyme (E), calculated from a molecular weight of 31,100 (see next paragraph), was 3.2×10^{-6} μmol. Thus, the molecular activity (V_{max}/E), which represents the maximum number of $\beta(1 \rightarrow 4)$ xylosidic bonds cleaved per enzyme molecule per minute, was 1.4×10^5 min^{-1}, assuming only one active site.

Molecular weight and amino acid analysis. Electrophoretic mobilities of protein standards on SDS-gels were plotted against their molecular weights to establish the standard line shown in Fig. 6. The electrophoretic mobility of purified xylanase A indicated that its molecular weight was about 33,000, and its amino acid composition (Table 2) gave a molecular weight of 31,100.

Dissociation of carbohydrates from the purified xylanase A. Three samples of purified xylanase A were tested for carbohydrate content by the phenol-sulfuric acid method (5). They contained 37.5, 70, and 111 mol of carbohydrate estimated as glucose per mol of protein. The variability of carbohydrate content and the fact that almost all of the molecular weight determined by the SDS-gel electrophoresis could be accounted for by the amino acid analysis indicated that xylanase A was not a true glycoprotein. We suspected that sugars, instead of being covalently bound to the enzyme, were tightly adsorbed. To dissociate the complex, a sample of purified enzyme was boiled with 1% SDS solution (pH 7.0) for 5 min to unfold the protein structure and allow any sugars to desorb. The denatured sample was passed through a Sephadex G-50 column equilibrated with 1% SDS to separate the enzyme from the sugars. SDS was then removed from the enzyme solution by the anion-exchange slurry method of Weber and Kuter (36). The resulting lyophilized enzyme preparation contained no carbohydrate, indicating that the original enzyme is not a glycoprotein. The specific xylanase activity recovery was 20%.

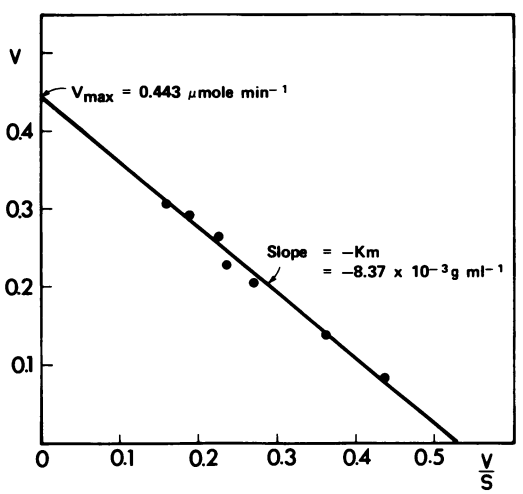

FIG. 5. *Eadie-Hofstee plot for determining K_m and V_{max} values for soluble larch xylan and purified xylanase A. The units of V are micromoles of reducing sugar (as xylose) released per minute under standard assay conditions. The units of S are percent xylan. The amount of enzyme used in each assay was 3.2×10^{-6} μmol.*

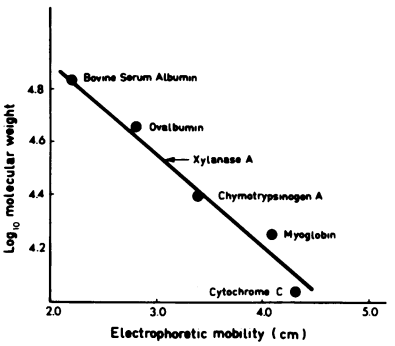

FIG. 6. *Estimation of molecular weight of xylanase A from a plot of log_{10} molecular weight against electrophoretic mobility on SDS-polyacrylamide gel.*

TABLE 2. *Amino acid composition of xylanase A*

Amino acid	No. of residues	Closest integer
Aspartic acid	31.58	32
Threonine	29.68	30
Serine	37.52	38
Glutamic acid	19.03	19
Proline	13.74	14
Glycine	47.00	47
Alanine	20.92	21
Valine	14.40	14
Methionine	1.44	2
Isoleucine	11.66	12
Leucine	11.00	11
Tyrosine	23.02	23
Phenylalanine	4.96	5
Histidine	4.14	4
Lysine	7.12	7
Arginine	5.81	6
Half-cystine	3.95	4
Tryptophan	8.50	9

Amino-terminal sequence. The amino-terminal amino acid sequence of xylanase A, presented in Table 3, appeared to reflect the overall

TABLE 3. *Automated*[a] *sequence analysis of xylanase A*[b]

Step of Edman degradation	Residue identified by hydroiodic acid hydrolysis	Residue identified by NaOH/$Na_2S_2O_4$ hydrolysis	Yield (nmol)	Residue identified by thin-layer and gas-liquid chromatography of PTH derivatives
1	Ala[c]		73	Ser
2	Gly		76	Gly
3	Abu[d]		61	Thr
4	Pro		23	Pro
5	Ala[c]		59	Ser
6	Ala[c]		53	Ser
7	Abu[d]		56	Thr
8	Gly		39	Gly
9	Abu[d]		46	Thr
10	Asx		44	Asp
11	Gly		37	Gly
12	Gly		27	Gly
13	Tyr		46	Tyr
14	Tyr		42	Tyr
15	Tyr		39	Tyr
16	Ala[c]		33	Ser
17		Trp	48	Trp
18		Trp	45	Trp
19	Abu[d]		22	Thr
20	Asx		19	Asp
21	Gly		17	Gly
22	Ala		25	Ala
23	Gly		20	Gly
24				Asp
25				Ala
26	Abu[d]		9	Thr
27				Tyr

[a] Beckman sequencer program 050972 with 1 M Quadrol.

[b] 150 nmol used in sequence analysis which was performed once.

[c] Ala is hydrolysis product of phenylthiohydantoin (PTH) derivative of Ser.

[d] α-Amino butyric acid (Abu) is hydrolysis product of PTH-Thr.

amino acid analysis in having a high aspartic acid and aromatic amino acid content. The sequence showed no homology with known sequences deposited in the Atlas of Protein Sequence and Structure data base (Washington, D.C.).

DISCUSSION

Xylanase A appears to be identical to a xylanase previously isolated from *S. commune* by preparative polyacrylamide gel electrophoresis (35). Both enzymes show similar pH and temperature optima and chromatographic properties; neither is a glycoprotein. Xylanase A hydrolyzes the β(1 → 4) linkage of xylan chains with remarkable efficiency; the molecular activity is only about 100 times less than that of the most active enzyme known, carbonic anhydrase (18), and is approximately equal to that of α-amylase (33).

Xylanase A exhibits some affinity for Sephadex gels, as evidenced from gel permeation chromatography. A globular protein of molecular weight 31,000 would normally elute at the void volume of Sephadex G-50. Adsorption on Sephadex gels has also been observed with other xylanases (29).

Wood-saccharifying microorganisms often produce large numbers of hydrolases with similar specificities. Eriksson and Pettersson found five cellulases with different amino acid compositions in *Sporotrichum pulverulentum* (9). Gum and Brown (11) concluded that the multiplicity of β(1 → 4)-D-glucancellobiohydrolases from *Trichoderma viride* is due to differentially glycosylated forms of the same polypeptide. Nakayama et al. (25) indicated that partial proteolysis is a factor in the multiple endocellulase system from the same microorganism. Another factor producing electrophoretic heterogeneity could be the complexing of enzyme and polysaccharide, which was observed with a cellulase from *Myrothecium verrucaria* (14) and with the previously isolated xylanase from *S. commune* after mixing homogeneous xylanase and xylan (35). Our studies confirm that xylanase A strongly binds carbohydrate in a noncovalent manner. After complete removal of the carbohydrate, about 20% of the xylanase activity was retained. This shows that the carbohydrate is not absolutely essential for the enzyme activity. It is possible that the carbohydrate is required for full activity, although a more likely explanation of the 80% loss in activity is a partial denaturation of the enzyme activity during boiling with SDS.

Despite widespread interest in xylanases and cellulases, their catalytic mechanisms have not been elucidated. It is tempting to speculate that both xylanases and cellulases use catalytic mechanisms similar to that of lysozyme, since the substrates of all of these enzymes are β(1 → 4)-linked pyranosides and since some highly purified enzymes possess both xylanase and cellulase activities (31, 34). Hurst et al. (13) recently determined that, in striking similarity to lysozyme, two carboxylate groups with respective pK's 4.0 to 4.5 and 5.0 to 5.5 are essential for the activity of a cellulase from *Aspergillus niger*.

Xylanases have been isolated from many microorganisms, and in three cases (8, 10; R. Toman, personal communication) amino acid analyses have been performed. Although these three enzymes were glycoproteins, they all contained

higher than average numbers of aromatic amino acid residues, a feature shared by xylanase A from *S. commune* and by lysozyme. We used the amino acid analyses to calculate an index of sequence identity by the method of Cornish-Bowden (3). Disappointingly, the index indicated that extensive homologies between any of the four xylanases are unlikely. The index also indicated no obvious homology between the *S. commune* xylanase A, various cellulases (9, 11), and lysozyme. The partial amino acid sequence of xylanase A is at present the only piece of sequence information available for any xylanase or cellulase. The partial sequence shows no homology with the sequence of lysozyme or any other known protein sequence. However, this does not rule out the possibility that a homology exists in the still unsequenced part of the xylanase.

To resolve the question of possible homology among xylanases, cellulases, and lysozyme, more information concerning the structure of the first two is required. Complete amino acid sequencing of xylanase A would, for example, conclusively determine whether the enzyme is homologous with lysozyme and thus whether the lysozyme catalytic mechanism can be postulated for xylanase A. *S. commune* has now been shown to be a source of xylanase suitable for such sequencing studies.

ACKNOWLEDGMENTS

We thank J. M. Macleod for the gas chromatographic analysis, G. O. Aspinall for a sample of xylobiose, H. I. Bolker for helpful discussion, M. Natriss for the amino acid analysis, and C. Maine for technical assistance.

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