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Purification and Characterization of a New Xylanase (APX-II) from the Fungus *Aureobasidium pullulans* Y-2311-1

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Aureobasidium pullulans **Y-2311-1 produced four major xylanases (EC 3.2.1.8) with pI values of 4.0, 7.3, 7.9, and 9.4 as revealed by isoelectric focusing and zymogram analysis when grown for 4 days on 1.0% oat spelt xylan. The enzyme with a pI of 9.4 was purified by ammonium sulfate precipitation, chromatography on a DEAE-Sephadex A-50 column, and gel filtration with a Sephadex G-75 column. The enzyme had a mass of about 25 kDa as determined by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration chromatography. The purified enzyme had a K_m of 7.6 mg · ml^{-1} and a V_{max} of 2,650 μmol · min^{-1} · mg^{-1} for birchwood xylan at 28°C and pH 4.5. It lacked activity towards carboxymethylcellulose, cellobiose, starch, mannan, *p*-nitrophenyl (pNP)-β-D-xylopyranoside, pNP-β-D-glucopyranoside, pNP-α-D-glucopyranoside, pNP-β-D-cellobioside, pNP-β-D-fucopyranoside, or pNP-α-D-galactopyranoside. The predominant end products of birchwood xylan or xylohexaose hydrolysis were xylobiose and xylose. The enzyme had the highest activity at pH 4.8 and 54°C. Sixty percent of the activity remained after the enzyme had been incubated at 55°C and pH 4.5 for 30 min. The sequence of the first 68 amino acid residues at the amino terminus showed homology to those of several other xylanases. Immunoblot analysis with antiserum raised against the purified xylanase revealed that two immunologically related polypeptides of 25 and 22 kDa were produced in *A. pullulans* cultures containing oat spelt xylan or xylose as carbon sources but not in cultures containing glycerol or glucose.**

The major structure of hemicellulose is xylan, which is a polymer of β-1,4-linked xyloses with arabinosyl and/or 4-*O*-methylglucurosyl side chains (30). The enzymatic degradation of xylan to xylose requires the catalysis of both endoxylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37). Potential applications of xylanase in biotechnology include biopulping wood (4, 6), pulp bleaching (10, 11, 25), treating animal feed to increase digestibility (31), processing food to increase clarification (1, 3), and converting lignocellulosic substances into feedstocks and fuels (4, 9).

It is characteristic that most xylanolytic microorganisms produce multiple xylanases with different physicochemical properties (31). The fungus *Aureobasidium pullulans* Y-2311-1 was shown to be among the most proficient of the xylan-degrading fungi, secreting extremely high levels of xylanolytic enzymes into culture media (20, 21). D-Xylose, xylobiose, xylan, and arabinose all induced, while glucose repressed, xylanase activity (20). Leathers (17) showed that two xylanases with similar molecular masses were secreted into the culture supernatant by *A. pullulans* grown on xylan or xylose, and one of these, which we designated APX-I and which had extremely high specific activity towards oat spelt xylan (OSX), was purified (19). In this paper, we report the purification and characterization of a second xylanase (APX-II) from *A. pullulans* Y-2311-1.

MATERIALS AND METHODS

Chemicals. Reagents for the cultivation of *A. pullulans* Y-2311-1 were purchased from Difco Laboratories (Detroit, Mich.). Resins for liquid chromatography and gel filtration calibration markers were purchased from Pharmacia LKB Biotechnology (Piscataway, N.J.). Reagents and standard markers for gel electrophoresis and immunoblot analysis were purchased from Bio-Rad Laboratories (Richmond, Calif.). Immobilon-P membranes for immunoblot analysis were obtained from Millipore Corp. (Bedford, Mass.). All other chemicals were products of Sigma Chemical Co. (St. Louis, Mo.).

Strain and cultivation conditions. *A. pullulans* Y-2311-1 was grown in YM medium (21) at 28°C with shaking (200 rpm). The medium contained 1.0% (wt/vol) OSX, D-xylose, glucose, or glycerol as the carbon source. Culture volumes were either 50 or 500 ml. For time course studies, cultures were sampled sterilely. The samples were stored frozen at −20°C until analyzed. No loss of xylanase activity at −20°C was observed over a period of 1 year.

Enzyme and protein assays. Birchwood xylan (BWX) (1.0% [wt/vol]) was prepared by solubilizing 1 g of BWX in 20 ml of 0.2 M NaOH. This solution was then adjusted to pH 4.5 by the addition of acetic acid, and the volume was adjusted to 100 ml with H_2O. Xylanase assays were performed by incubating 500 μl of BWX with 50 μl of enzyme solution at 28°C for 15 min. Other substrates (1% or 5 mM) were tested under the same conditions. Reactions were terminated by the addition of either 3,5-dinitrosalicylic acid reagent for natural substrates or 1 M Na_2CO_3 for synthetic substrates. Reducing sugars were measured with the 3,5-dinitrosalicylic acid reagent (23) with D-xylose as a standard. *p*-Nitrophenol released from synthetic substrates was measured by the A_{400} increase. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of xylose equivalent or *p*-nitrophenol per min. Protein was measured by the procedure of Lowry et al. (22) or with the bicinchoninic acid microprotein assay kit from Pierce (Rockford, Ill.).

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Enzyme purification. Purification of the xylanase was monitored by the enrichment of a protein band with a pI value of 9.4 on isoelectric focusing (IEF) gels, by a 25-kDa protein band detected on sodium dodecyl sulfate (SDS)-polyacrylamide gels, and by an increase in xylanase specific activity. One liter of a 4-day-old culture grown on 1% (wt/vol) OSX as the carbon source was the starting material. *A. pullulans* cells and residual OSX were removed by centrifugation at 5,000 × *g* for 5 min, and the supernatant was treated with ammonium sulfate. Proteins which precipitated in the range between 30 and 50% saturation contained about 20% of the total xylanase activity. The precipitate was collected by centrifugation at 10,000 × *g* for 20 min and dialyzed against 50 mM sodium phosphate buffer, pH 6.8. The sample was further concentrated by tangential-flow ultrafiltration (Amicon Inc., Beverly, Mass.) against a membrane (YM3) having an apparent molecular-weight cutoff of 3,000. The concentrated enzyme sample (12 ml) was loaded onto a DEAE-Sephadex A-50 column (3.5 by 20 cm) which had been equilibrated with 50 mM sodium phosphate buffer, pH 6.8. Proteins were eluted with 500 ml each of 50 mM sodium phosphate, pH 6.8, containing 0, 0.5, or 1.0 M NaCl. Fractions (2.5 ml) were collected at a flow rate of 0.5 ml · min^{-1}, and those with high xylanase activity were pooled. Desalting and equilibration of the pooled enzyme against 50 mM acetate buffer, pH 4.5, was again achieved by using tangential-flow ultrafiltration. The enzyme preparation was loaded onto a G-75 gel filtration column (2 by 85 cm), and fractions of 2.5 ml were collected at a flow rate of 0.5 ml · min^{-1}. Bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa) (Pharmacia) were used as standards to calibrate the column.

Antiserum production. Purified xylanase (100 μg in 100 μl of 50 mM sodium acetate buffer, pH 4.5) was emulsified with an equal volume of Hunter's TiterMax adjuvant (CytRx, Atlanta, Ga.) and injected into a 4-month-old rabbit. Blood (2 ml) was collected before the injection and once every week after the injection. Antibody titer was determined by enzyme-linked immunosorbent assay. When the antibody titer reached an adequate level (6 weeks after the injection), 20 ml of blood was drawn every week for 4 more weeks.

SDS-PAGE, immunoblot analysis, and IEF. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (15). The acrylamide concentration in gels was 12.5% (wt/vol). Protein bands in the gels were either visualized by Coomassie brilliant blue R-250 staining or subjected to immunoblot analysis. Transfer of proteins from gels to Immobilon-P membranes was done for 1 h at 4°C in 20 mM Tris-HCl (pH 8.3)–20% (vol/vol) methanol–0.1% (wt/vol) SDS with a Mini Trans-Blot cell (Bio-Rad). Detection of protein bands with xylanase antiserum was performed with a horseradish peroxidase immunoblot kit (Bio-Rad) according to the manufacturer's instructions. IEF was performed as described by Sterjiades et al. (28) on precast gels with pHs of 3 to 10 (Precote; Serva Biochemicals). Serva protein test mixture 9 was used as a standard for determining pI values. After focusing had been performed, proteins were detected by Coomassie brilliant blue R-250 staining and xylanase activity was detected by zymogram analysis (26). For zymogram analysis, a gel (0.75 mm thick) containing 0.2% remazol brilliant blue *R*-D-xylan, 1% agarose, and 100 mM sodium acetate, pH 4.5, was overlaid on the focusing gel plate. After incubation of the gels at 28°C for 15 min, the overlay gel was removed and immersed in a solution of 50% (vol/vol) ethanol and 50 mM sodium acetate, pH 5.0, for incubation overnight at 4°C. Xylanase activity was visualized as clear bands against a blue background. Each lane of the original IEF gel was also sliced into 0.25-cm pieces. Each piece was soaked overnight in 0.5 ml of 100 mM sodium acetate, pH 4.5, and eluted xylanase activity was assayed under standard conditions.

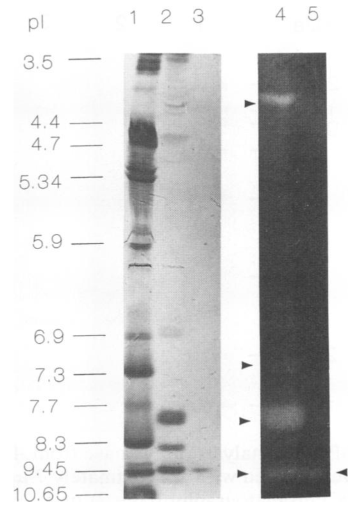

FIG. 1. IEF of xylanase samples from *A. pullulans*. Supernatant proteins (20 μg, lanes 2 and 4) and purified xylanase (3 μg, lanes 3 and 5) were loaded onto precast gels. After focusing had been performed, gel plates were subjected to Coomassie brilliant blue staining (lanes 1, 2, and 3) or xylanase zymogram analysis (lanes 4 and 5). Activity bands on the zymogram gel, difficult to see, are indicated by arrows. pI markers (lane 1) included amyloglucosidase (3.5), feritin (4.4), bovine albumin (4.7), β-lactoglobulin (5.34), conalbumin (5.9), horse myoglobin (7.3), whale myoglobin (8.3), ribonuclease (9.45), and cytochrome *c* (10.65).

Amino acid analysis and amino-terminal sequencing. Amino acid analysis of the purified xylanase (0.4 μg) was performed on a 120A amino acid analyzer (Applied Biosystems Inc., Foster City, Calif.). The amino (N)-terminal amino acid sequencing was performed on an Applied Biosystems 477A gas phase sequencer equipped with an automatic on-line phenylthiohydantoin derivative analyzer. Homologous peptide sequences were searched for and aligned with the N-terminal residues of the purified xylanase by using the Genetics Computer Group programs of the University of Wisconsin at the University of Georgia bioscience computing facility.

TLC. Thin-layer chromatography (TLC) on silica gel plates (Analtech, Inc., Newark, Del.) was used to analyze the hydrolysis products of xylanases. Alkaline-solubilized BWX (1% [wt/vol]) or xylohexaose (10 mM) in 50 mM sodium acetate (pH 4.5) was incubated with 200 U of purified xylanase or crude supernatant enzyme samples at 28°C. Hydrolysis was stopped by heating the reaction solution at 80°C for 10 min. Aliquots (10 μl) were spotted onto a TLC plate and then partitioned for 2.5 h at room temperature with chloroform-glacial acetic acid-H_2O (6:7:1 [vol/vol]). Sugars were visualized by diphenylamine staining as described by Lake and Goodwin (16).

RESULTS

The crude supernatant samples of *A. pullulans* culture were subjected to IEF (Fig. 1). More than 10 protein bands

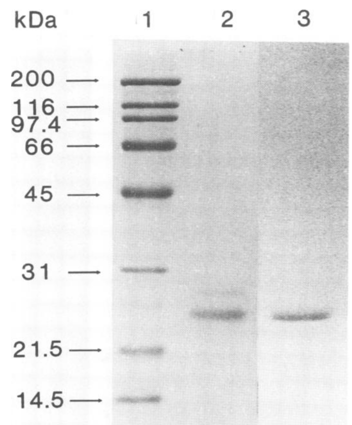

FIG. 2. SDS-PAGE analysis of xylanase from *A. pullulans*. Gels (12% [wt/vol]) were loaded with approximately 7 μg of protein after DEAE-Sephadex chromatography (lane 2) or 5 μg of protein after gel filtration (lane 3). Molecular mass markers (lane 1) included myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

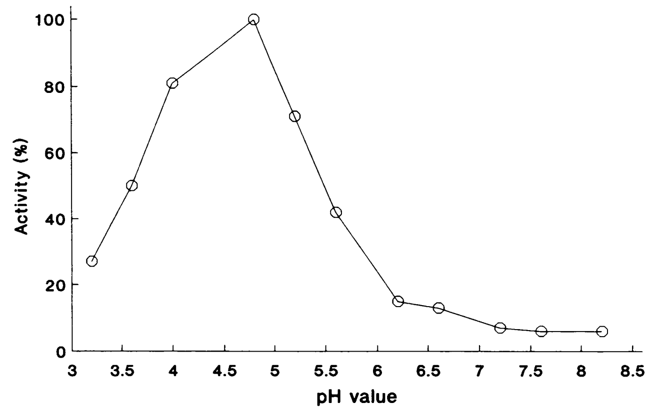

FIG. 3. Effect of pH on the activity of purified xylanase. The activity of the purified enzyme was measured under the conditions described in Materials and Methods, except that the pH of the reaction mixture was adjusted with either 0.1 M sodium acetate buffer (pH 3.1 to 5.4) or 0.1 M sodium phosphate buffer (pH 5.8 to 8.2).

were seen after Coomassie blue staining. Four of these bands with pI values of 4.0, 7.3, 7.9, and 9.4 exhibited xylanase activity. Elution of the xylanase-active bands from the IEF gel showed that these bands contained over 80% of the total activity applied to the gel, distributed as follows: pI value of 4.0, 12%; pI value of 7.3, 6%; pI value of 7.9, 36%; and pI value of 9.4, 30%.

The xylanase with a pI value of 9.4 was purified to apparent homogeneity. This enzyme, designated APX-II, had a mass of 25,000 Da on SDS-PAGE (Fig. 2). The specific activity (2,440 U · mg^{-1}) of the purified protein (Table 1) was slightly higher than that (2,100 U · mg^{-1}) reported for xylanase APX-I (19). After the ammonium sulfate precipitation with 30 to 50% saturation, the 25-kDa band was substantially enriched while the other xylanases stayed in the supernatant. Specific activity of the enzyme preparation increased 3.6-fold during this step. Chromatography on a DEAE-Sephadex column resulted in one peak of xylanase activity which eluted in the flowthrough volume. Chromatography on a Sephadex G-75 column, the final step used for the xylanase purification, again resulted in only one peak of xylanase activity. A polypeptide band of about 27 kDa on SDS-PAGE was completely removed during the gel filtration step, even though the specific activity of the enzyme preparation did not increase dramatically (Fig. 2). The xylanase peak eluted with an apparent mass of 22 to 25 kDa. The enzyme pooled from peak fractions after gel filtration migrated as a single 25-kDa band on SDS-PAGE stained with Coomassie brilliant blue (Fig. 2).

TABLE 1. Purification of xylanase from *A. pullulans*[a]

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)
Culture filtrate	349.3	146,000	417	1
$(NH_4)_2SO_4$ precipitation	18.5	28,000	1,520	3.6
DEAE-Sephadex A-50	15.7	26,200	1,670	4.0
Sephadex G-75	6.2	15,100	2,440	5.8

[a] This purification was from 1 liter of culture grown for 4 days with 1% OSX as the substrate.

The purified xylanase was tested for the effect of temperature and pH on the activity. The initial activity of this enzyme towards BWX was highest at pH 4.8, and at least 80% of the maximal rate was attained from pH 3.8 to pH 5.4 (Fig. 3). The purified xylanase gave the highest initial activity towards BWX at a temperature of 54°C in 50 mM sodium acetate, pH 4.5. Under these conditions, 50% of the highest activity was obtained at 25 and 62°C (data not shown). The enzyme was fairly stable at temperatures up to 50°C in 50 mM sodium acetate, pH 4.5 (Fig. 4). After the enzyme was preincubated at 50°C in 50 mM sodium acetate, pH 4.5, for 4 h, 73% of the activity was retained. Sixty percent of the activity remained after the enzyme was preincubated at 55°C for 30 min. This enzyme, however, was not stable at 60°C. Rapid inactivation of enzyme activity was observed at this temperature.

The purified enzyme was assayed for hydrolytic activity against a variety of natural and synthetic substrates. BWX and OSX were hydrolyzed at similar rates at 28°C and pH 4.5. Under these conditions, no detectable activity towards carboxymethylcellulose, cellobiose, starch, mannan, *p*-nitrophenyl (pNP)-β-D-xylopyranoside, pNP-β-D-glucopyranoside, pNP-α-D-glucopyranoside, pNP-β-D-cellobioside, pNP-β-D-fucopyranoside, or pNP-α-D-galactopyranoside was observed. A Lineweaver-Burk plot of the activity over a broad range of BWX concentrations (0.2 to 12.0 mg · ml^{-1}) showed that the K_m and V_{max} of this enzyme were 7.2 mg of xylan · ml^{-1} and 2,650 μmol · min^{-1} · mg^{-1} of protein, respectively, at 28°C and pH 4.5.

Hydrolysis products released by the purified xylanase from xylohexaose and OSX were separated by TLC (Fig. 5). The predominant end products from OSX hydrolysis were xylobiose and xylose, even though xylotriose was initially produced. This suggested that xylotriose was produced as an intermediate that was eventually cleaved to xylobiose and xylose. It appeared that xylopentaose was also produced from OSX, and this product did not disappear with increasing time. No arabinose was detected among the hydrolysis products of OSX. Xylobiose and xylotriose were rapidly

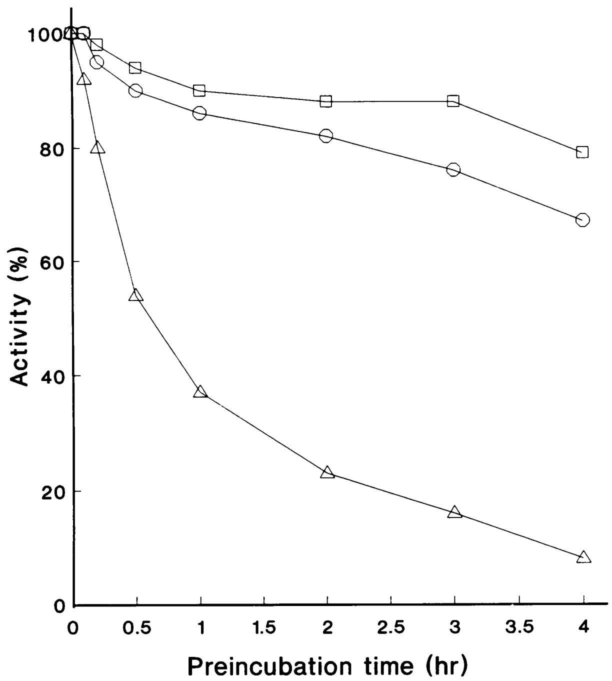

FIG. 4. Thermostability of purified xylanase. Purified xylanase in 50 mM sodium acetate, pH 4.5, was preincubated at 40°C (□), 50°C (○), or 60°C (△), and aliquots were withdrawn over time for assay under the standard conditions.

generated from xylohexaose, and xylotriose was again further hydrolyzed to xylobiose and xylose.

Antiserum was raised against the purified xylanase, and immunoblot analysis of xylanase production by cultures grown for 3 days on various substrates was performed. A band with an apparent M_r of 25,000 was found in lanes loaded with purified enzyme and with OSX- and D-xylose-grown cultures, but not in lanes loaded with samples from glucose- or glycerol-grown cultures (Fig. 6A). A high-mass (above 100-kDa) band which was cross-reactive with the antiserum was present in all supernatants, regardless of the

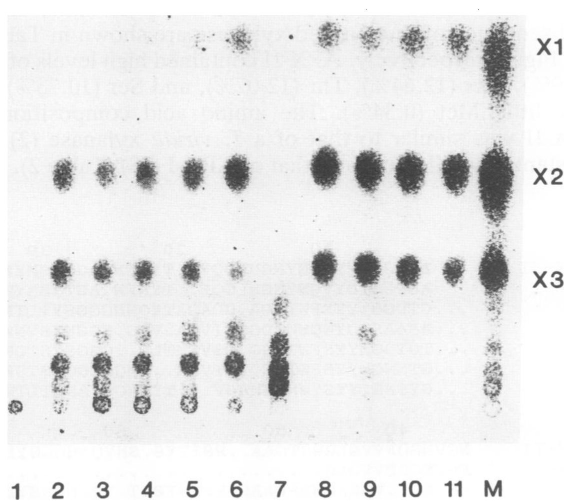

FIG. 5. TLC analysis of OSX and xylohexaose hydrolysis products released by purified xylanase. Enzyme (200 U) was incubated in 50 mM sodium acetate, pH 4.5, at 28°C with 1% (wt/vol) solubilized OSX for 0 (lane 1), 10 (lane 2), and 30 (lane 3) min and 1.5 (lane 4), 6 (lane 5), and 24 (lane 6) h or with 10 mM xylohexaose at 0 (lane 7), 10 (lane 8), and 30 (lane 9) min and 1.5 (lane 10) and 6 (lane 11) h. Oligoxylosaccharide standards (lane M) were run under the same conditions. X1, xylose; X2, xylobiose; X3, xylotriose.

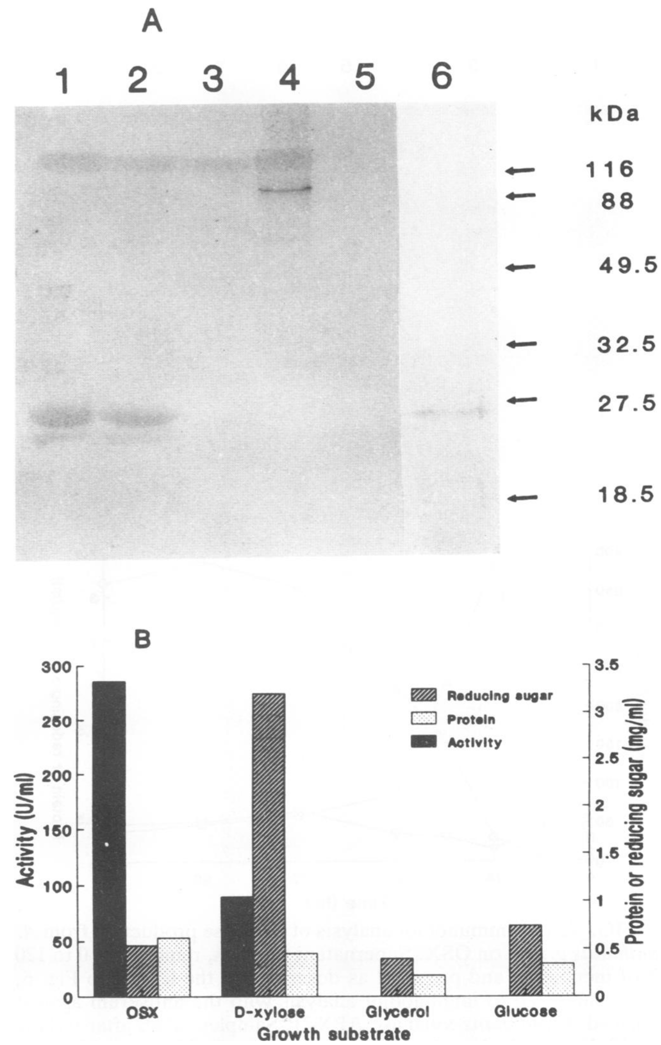

FIG. 6. (A) Immunoblot analysis of xylanase production from *A. pullulans* grown on various substrates. Cultures were incubated at 28°C for 72 h with shaking (150 rpm). Cells and residual insoluble substrates were removed by centrifugation at 8,000 × *g* for 5 min, and supernatants were concentrated 10-fold by ultrafiltration (Centricon-3; Amicon). Aliquots (10 μl) of concentrated supernatants from cultures grown on OSX (lane 1), xylose (lane 2), glycerol (lane 3), or glucose (lane 4), as well as *T. viride* xylanase (40 U; Sigma) (lane 5) and the purified *A. pullulans* xylanase (2 μg) (lane 6), were analyzed by immunoblotting with the antiserum against purified *A. pullulans* xylanase APX-II. Migrations of prestained SDS-PAGE protein standards (Bio-Rad), including myosin (205 kDa), β-galactosidase (116.5 kDa), phosphorylase B (106 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa), and lysozyme (18.5 kDa) are indicated on the right. (B) Levels of xylanase activity, as well as protein and reducing sugars in the culture supernatants used for immunoblot assays, are shown.

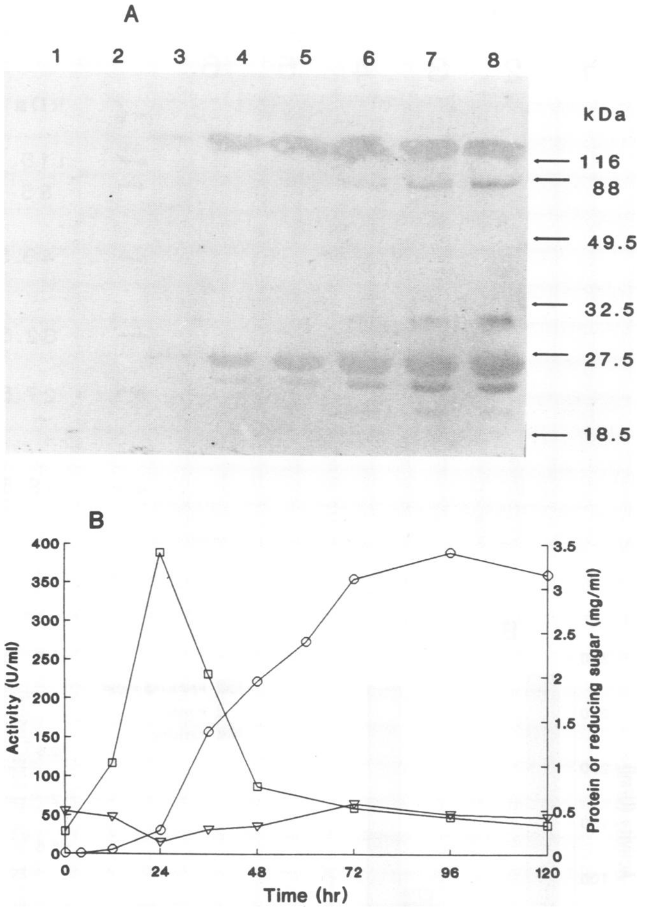

FIG. 7. (A) Immunoblot analysis of xylanase production from *A. pullulans* grown on OSX. Supernatant samples, removed at 0 to 120 h of incubation and prepared as described in the legend to Fig. 6, were subjected to immunoblot analysis with the antiserum against purified *A. pullulans* xylanase APX-II. Samples taken after 0 (lane 1), 12 (lane 2), 24 (lane 3), 36 (lane 4), 48 (lane 5), 72 (lane 6), 96 (lane 7), and 120 (lane 8) h of incubation were analyzed. (B) Xylanase activity (○) and protein (▽) and reducing sugar (□) concentrations are shown.

carbon source. A 22-kDa cross-reactive band was detected in OSX- and xylose-grown cultures but not in those grown in glucose or glycerol. Supernatant xylanase preparation from a *Trichoderma viride* (2) culture gave a very faint cross-reactive band of about 20 kDa. Xylanase activity and protein and reducing sugar concentrations for the samples used in the immunoblot analysis are shown in Fig. 6B. Xylanase activity was high in OSX-grown (285 U · ml^{-1}) and xylose-grown (91 U · ml^{-1}) cultures but was not detectable in glycerol- or glucose-grown cultures. Xylanase activity levels and the intensity of the 25- and 22-kDa bands on immunoblot membranes were highly correlated. When concentrated OSX culture supernatants were compared over 120 h of incubation time by immunoblot analysis, the 25-kDa xylanase was detected as early as 12 h after inoculation and subsequently increased continuously up to 96 h (Fig. 7A). The 22-kDa protein appeared in the supernatant 24 h after inoculation and increased steadily over the remaining 120 h. The high-molecular-mass (about 120-kDa) cross-reactive protein first appeared at 24 h and did not change substantially afterwards (Fig. 7A). Other minor cross-reactive bands

TABLE 2. Amino acid composition of xylanases

Amino acid	mol% amino acid for xylanase[a]		
	A. pullulans		*T. viride*
	APX-II	APX-I	
Ala	7.78	11.38	3.9
Arg	3.09	6.61	3.7
Asx	12.64	12.81	14.3
Cys	0	0	0
Glx	8.29	14.58	5.0
Gly	15.90	3.93	14.6
His	3.04	0	1.8
Ile	2.35	4.59	4.2
Leu	2.73	0.62	3.8
Lys	2.17	16.34	1.6
Met	0.34	0	0
Phe	3.11	5.54	4.3
Pro	2.40	1.96	3.3
Ser	10.78	13.24	11.8
Thr	12.07	5.73	7.7
Trp	ND[b]	ND	3.9
Tyr	7.19	0.85	8.7
Val	6.42	0	7.2

[a] Data for APX-I and the *T. viride* xylanase are from references 18 and 2, respectively.
[b] ND, not determined.

(82 and 30 kDa) were also noted in the supernatant at the late stages of incubation. The xylanase activity levels in the supernatant increased continuously up to 385 U · ml^{-1} after 96 h of incubation (Fig. 7B), which matched very well the increase in intensity of the 25- and 22-kDa bands on the immunoblot (Fig. 7A). Reducing sugars in OSX culture increased to 3.4 mg · ml^{-1} in the early period of incubation (up to 24 h) and decreased rapidly to a low level (about 0.5 mg · ml^{-1}). Protein concentration remained relatively stable over the period of incubation, though small changes were recorded (Fig. 7B).

The amino acid composition and the N-terminal amino acid sequence of the purified xylanase are shown in Table 2 and Fig. 8, respectively. APX-II contained high levels of Gly (15.9%), Asx (12.64%), Thr (12.07%), and Ser (10.78%) but very little Met (0.34%). The amino acid composition of APX-II was similar to that of a *T. viride* xylanase (2) but substantially different from that of APX-I (18) (Table 2). The

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                10          20          30
APX-II     AGPGGINYVQNYNGNLGQF..TYNEN.AGTYSMYW.N
APX-I      AGPGGIDYVQNYNGNLGQF..TYNEN.AGTYSMYW.N
Sc      ...GTDGGYYYSFWTDGA.GDADATYQNNGGGSYTLTWSG
Bs      ...ASAASTDYWQNWTDGGGIVNA.VNG.SGGNYSVNW.G
SlC     ...TGTDGMYYSFWTDGG.GSVSMTLN..GGGSYSTQW.T
SlB     ...GTNNGYYYSFWTDSQ.GTVSM..NMGSGGQYSTSW.R
Th      ...GYSNG.YYSYWNDGHAGV...TYTNGGFANATLTW.S

           40        50           60
APX-II  NGVNGDFVVGLGWSTGAA..RSITYS.SNYQASGGSYL...
APX-I   NGVNGDFVVGLG.............................
Sc      NNKNL..VGGKGWNPGAAS.RSISYSGT.YQPNGNSYL...
Bs      N..TGNFVVGKGWTTGSP.FRTINYNAGVWAPNGNGYL...
SlC     NC..GNFVAGKGWSTGDGNVR...YNGY.FNPVGNGYG...
SlB     N..TGNFVAGKGWANGGR..RTVQYSGS.FNTSGNAYL...
Th      N..SGNFVGGKGWQPGT.KNKVINFSGS.YNPNGNSYL...
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FIG. 8. Alignment of N-terminal amino acid sequence of APX-II with homologous xylanase sequences of *A. pullulans* (APX-I), *S. commune* (Sc), *B. subtilis* (Bs), *S. lividans* C (SlC) and B (SlB), and *T. harzanium* (Th). Aligned residues which match those found in APX-II are shown in boldface type.

first 45 residues at the N terminus of APX-I and APX-II were the same, except that APX-II had Asn instead of Asp at position 7 (Fig. 8). Sequences homologous to the first 68 amino acid residues at the amino terminus of APX-II were searched for and retrieved from the Swissprot data bank. The sequences included corresponding regions of xylanases from *Schizophyllum commune* (45.6%), *Bacillus subtilis* (38.7%), *Streptomyces lividans* C (36.8%) and B (35.3%), and *Trichoderma harzanium* (32.4%) (Fig. 8).

DISCUSSION

A new xylanase (APX-II) possessing high activity towards BWX has been purified from the culture supernatant of *A. pullulans* Y-2311-1. The V_{max} of this enzyme (2,650 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1}) as determined from Lineweaver-Burk plots is among the highest reported. The exceptionally high specific activity of this enzyme might be an attractive property for biotechnological application of this enzyme. In fact, unfractionated preparations of this enzyme have been successfully used in the biobleaching of pulp at experimental scales by our research group (32).

The low apparent mass (25 kDa) and the high pI value (9.4) of this enzyme are similar to those of xylanases from *Streptomyces thermoviolaceus* OPC-520 (33 kDa, pI value of 8.0) (29), *Robillarda* sp. Y-20 (17.6 kDa, pI value of 9.7) (13), *S. lividans* 66 (31 kDa, pI value of 8.4) (12), and *Streptomyces roseiscleroticus* (22.6 kDa, pI value of 9.5) (7). This group of xylanases has been assigned to the category of low-M_r, basic xylanases, in contrast to high-M_r, acidic xylanases (31). Most xylanolytic organisms produce both types of xylanases. IEF and zymogram analysis of culture supernatants from *A. pullulans* done in the present study revealed that both acidic (pI value of 4.0) and basic (pI values of 7.3, 7.9, and 9.4) xylanases are produced. The size of the acidic xylanase from this organism, however, is not yet known.

APX-II is specific for hydrolyzing natural xylan and is free of cellulase activity, which are desirable properties for biobleaching of pulps. Some xylanases have both xylanase and cellulase activities (27). Xylobiose and xylose are produced as end products, while higher oligoxylosaccharides appear to be produced only as intermediates of xylan hydrolysis by this enzyme. No free arabinose is produced from xylan by this enzyme. On the basis of these results, it is safe to say that APX-II is a typical endo-β-1,4-xylanase. In spite of the extremely high specific activity of this enzyme towards xylan, the K_m (7.6 mg $\cdot$ ml^{-1}) of this enzyme is similar to that of xylanases from other sources (7, 12, 13). The pH and temperature for optimal enzyme activity of this enzyme are also in the range of those reported for xylanases from other mesophilic fungi and bacteria (31).

Immunoblot analysis with antiserum against APX-II revealed that two protein bands of 25 and 22 kDa are synthesized in xylan- and xylose-grown but not in glucose- or glycerol-grown culture supernatants. The intensity of these two bands was closely correlated with the xylanase activity levels in the supernatants, but the other cross-reactive band (above 100 kDa) was not correlated with the activity levels. The results suggested that the synthesis of APX-II in *A. pullulans* might be regulated at the transcriptional level rather than at the translational or posttranslational level. Xylose, xylobiose, or their derivatives may be transported into the cell to trigger the transcription process.

The N terminus of APX-II is homologous to those of xylanases from several other fungi and bacteria. The APX-II N-terminal sequence showed the highest homology to that of a xylanase from *S. commune* but much lower homology to those of the xylanases from *T. harzanium* or *Cryptococcus albidus*. The homology of this sequence to those of xylanases from several bacteria, including *B. subtilis* and *S. lividans*, was intermediate. Tyr, Gly, Trp, Asn, Gly, Trp, Gly, and Tyr at positions 8, 17, 32, 34, 43, 46, 64, and 67 of APX-II, respectively, were aligned and conserved in all xylanases. Residues from positions 40 to 51 are highly conserved, and this region may be important for the function of the enzyme.

Although the relationship between APX-I and APX-II is unclear, APX-I (18, 19) and APX-II appear to be closely related isozymes. Purified APX-I was reported to have an M_r of 20,000 and a pI value of 8.5 (19), suggesting that APX-I might be the 22-kDa cross-reactive band on our immunoblots and the band with a pI value of 7.9 on our IEF gels. Although APX-I and APX-II have distinct physicochemical properties such as molecular mass, pI value, and amino acid composition, the first 45 N-terminal amino acids of the two enzymes are very similar. The only difference was that APX-II had Asn instead of Asp at position 7. These data suggest that two conserved xylanase genes might exist. However, APX-I and APX-II might be posttranslational derivatives of one another. This could be caused by glycosylation, proteolysis, or both. Two xylanases with identical N-terminal sequences encoded by distinct genes in *S. lividans* have been reported (27). Xylanases possessing the same polypeptide but with different glycosylations have been demonstrated (14). On the other hand, the pronounced differences in amino acid composition (Table 2) between APX-I and APX-II indicate that they are encoded by very different genes. To clarify the relationship, if any, between these two enzymes, more investigations need to be done.

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