J. Microbiol. Biotechnol. (2010), **20**(10), 1415–1423
doi: 10.4014/jmb.1003.03031
First published online 21 July 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Purification and Characterization of a Thermostable Xylanase from *Fomitopsis pinicola*

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Received: March 18, 2010 / Revised: June 2, 2010 / Accepted: June 23, 2010

An extracellular xylanase was purified to homogeneity by sequential chromatography of *Fomitopsis pinicola* culture supernatants on a DEAE-Sepharose column, a gel filtration column, and then on a MonoQ column with fast protein liquid chromatography. The relative molecular mass of the *F. pinicola* xylanase was determined to be 58 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis and by size-exclusion chromatography, indicating that the enzyme is a monomer. The hydrolytic activity of the xylanase had a pH optimum of 4.5 and a temperature optimum of 70°C. The enzyme showed a $t_{1/2}$ value of 33 h at 70°C and catalytic efficiency (k_{cat}=77.4 s^{-1}, k_{cat}/K_m=22.7 mg/ml/s) for oatspelt xylan. Its internal amino acid sequences showed a significant homology with hydrolases from glycoside hydrolase (GH) family 10, indicating that the *F. pinicola* xylanase is a member of GH family 10.

Keywords: *Fomitopsis pinicola*, glycoside hydrolase, purification, thermostability, xylanase

Xylans are the major hemicelluloses in differentiated hardwood; they represent an enormous reserve of utilizable biomass, as hardwoods consist of 25–32% hemicelluloses whereas softwoods contain only 15–25% hemicelluloses [3]. Hence, xylans represent a considerable reservoir of fixed carbon in nature [26]. Most of the xylanases characterized show optimal activity at slightly acidic pH values (pH 5.0–6.0) for most fungal xylanases, whereas pH values optima of bacterial xylanases are generally slightly higher [33]. Most of the xylanases characterized show optimal activity at temperatures between 40°C and 70°C. The xylanases and cellulases together with pectinases account for about 20% of the world enzyme market [29].

The xylans are complex and highly variable polysaccharides with a β-1,4-linked backbone of xylopyranosyl residues containing 4-*O*-methyl-glucoronosyl, 4-*O*-arabinosyl, and acetic acid side groups. Endo-1,4-β-D-xylanases (E.C. 3.2.1.8) are responsible for random cleavage of the xylan backbone and, hence, are industrially significant. Amino acid sequence comparisons of several endo-1,4-β-D-xylanases indicate that xylanases have been grouped mainly into two families of GH: GH10 and GH11 [15, 34]. However, other GH families, 5, 7, 8, and 43, have also been found to contain distinct catalytic domains with a demonstrated endo-1,4-β-D-xylanase activity [8]. Xylanases of family GH11 are of low molecular mass with pI 8–9.5 compared with the family GH10 that are of high molecular mass with lower pI values [6]. The known xylanolytic fungi are mostly ascomycetes. Particular attention has been granted to the enzymes produced by *Aspergillus* [10] and *Trichoderma* [31].

Brown-rot fungi primarily utilize the cellulose and hemicellulose components of wood biomass and then rapidly depolymerize the cellulose without removing the surrounding lignin that normally prevents microbial attack [19]. In this present study, xylanase-producing fungi were screened among 112 fungal strains, and *Fomitopsis pinicola* KMJ812 was found as a potent xylanase-producing strain. Under optimized conditions, *F. pinicola* produced xylanase with a 5.4 U/mg-protein specific activity. Here, we purified a novel xylanase from *F. pinicola* KMJ812 (KACC 93064P) to homogeneity and characterized its physiological and kinetic parameters. The properties of the enzyme, including its substrate specificity, molecular form, inhibition by product xylose, and partial amino acid sequence revealed that this enzyme is a xylanase, a member of GH family 10.

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Materials and Methods

Screening of Xylanase-Producing Strain

All fungal strains were obtained from the Korean Agricultural Culture Collection (KACC). Initial screening of xylanase-producing fungi was carried out in agar plates containing 0.4% Remazol Brilliant Blue xylan. Based on the zone of clearance observed, 15 strains were inoculated into 3 ml of the growth medium containing (g/l) peptone (8), yeast extract (2), KH_2PO_4 (5), K_2HPO_4 (5), $MgSO_4 \cdot 7H_2O$ (3), Thiamine·HCl (0.005), and oat spelt xylan (Sigma, MO, U.S.A.), and cultivated at 28°C with agitation at 200 rpm for 7 days. Xylanase activity of the culture broth was analyzed using 1% oatspelt xylan as the substrate. After analyses, the strain with the highest xylanase activity was selected.

Culture Conditions

For flask culture, the mycelia of *F. pinicola* KMJ812 were inoculated into 100 ml of potato dextrose broth. Precultures (5 ml) were inoculated into 200 ml of xylanolytic medium in a 1 l fermenter. This culture media contained (g/l) peptone (8), yeast extract (2), KH_2PO_4 (5), K_2HPO_4 (5), $MgSO_4 \cdot 7H_2O$ (3), ThiamineHCl (0.005), and oatspelt xylan. The effect of carbon or nitrogen source on xylanase production was investigated after 17 days of cultivation in flasks containing medium composed of 50 g/l of carbon sources and various nitrogen sources. The concentration of nitrogen source was adjusted to the same content of nitrogen using the Kjeldahl method. The medium used for xylanase production contained (g/l) peptone (8), yeast extract (2), KH_2PO_4 (5), K_2HPO_4 (5), $MgSO_4 \cdot 7H_2O$ (3), ThiamineHCl (0.005), and rice straw (20).

Enzyme Assay

Xylanase activity was assayed using 1% (w/v) oatspelt xylan as the substrate. Xylan was dissolved in 100 mM sodium acetate buffer (pH 5.0). The reaction mixture containing 10 μg of the enzyme and 2.5 mg of the substrate was incubated for 30 min at 50°C. The amount of the reducing sugars liberated was estimated following the 3,5-dinitrosalicylic acid (DNS) method [25]. Xylose was used as a standard. One unit of xylanase activity was expressed as μmole of reducing sugars (xylose equivalent) released in 1 min under the above conditions. Cellulase activity was assayed as above, using low-viscosity carboxymethylcellulose (1%) in place of xylan.

Purification of Xylanase

All procedures were performed at 4°C, and 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol (DTT) was used in the purification procedures unless otherwise stated. Protein was measured by the the Bradford method [4], using bovine serum albumin as a standard. Protein in the column effluents was monitored by measuring the absorbance at 280 nm. All chromatographic separations were performed using an ÄKTA FPLC system (ÄKTA, Sweden).

Step 1: Preparation of crude enzyme. Cells from the culture broth were harvested by centrifugation at 10,000 ×*g* for 30 min. After washing with 20 mM sodium acetate buffer (pH 5.0), the washes and supernatants were combined, concentrated, and desalted by ultrafiltration through a polyether sulfone membrane (30 kDa cutoff) in a stirred cell (Amicon, Beverly, MA, U.S.A.).

Step 2: DEAE-Sepharose chromatography. The dialyzed enzyme solution was loaded on a DEAE Sepharose Fast Flow column (1.6×10 cm; Amersham Biosciences) equilibrated with 20 mM sodium acetate buffer at pH 5.0, and proteins were eluted with a 180-min linear gradient of 0−0.5 M NaCl in the same buffer at a flow rate of 1.0 ml/min. Fractions of 1 ml each were collected and assayed for xylanase activity. Active fractions were pooled, dialyzed against the same buffer, and concentrated with ultrafiltration for further purification.

Step 3: Sephacryl gel filtration chromatography. The concentrated enzyme solution was loaded on a HiPrep 16/60 Sephacryl S-300 HR column (1.0 cm×120 cm; Amersham Biosciences) equilibrated with 20 mM sodium acetate buffer containing 100 mM NaCl at pH 5.0, and proteins were eluted with the same buffer at a flow rate of 0.5 ml/min. Active fractions were pooled, dialyzed against the same buffer, and concentrated with ultrafiltration.

Step 4: MonoQ ion-exchange chromatography. The enzyme was further purified with a MonoQ ion-exchange column 5/50 GL (1.0×10 cm; Amersham Biosciences) previously equilibrated with 20 mM sodium acetate buffer (pH 5.0). The enzyme was eluted with a 180-min linear gradient of 0−0.5 M NaCl in the same buffer at a flow rate of 0.5 ml/min. The combined active fractions were pooled, concentrated, and dialyzed against the same buffer and concentrated with a Centricon (Millipore, Bedford, MA, U.S.A.) ultrafiltration device with a molecular mass cutoff of 30 kDa, and then used as a purified enzyme in the following experiments.

Determination of pH and Temperature Optima

The optimal pH of xylanase activity was determined by incubating the purified enzyme at 50°C for 15 min in different buffers: citrate (100 mM, pH 3−4.5), sodium acetate (100 mM, pH 4.5−5.5), and phosphate (100 mM, pH 5.5−8). To determine the optimal temperature, the enzyme was incubated in sodium acetate buffer (100 mM, pH 5) for 15 min at different temperatures: from 25°C to 100°C. To determine the thermostability of xylanase activity, the purified enzyme was incubated at different temperatures (40°C−80°C) in the absence of substrate. After keeping them for certain periods of time (0−25 days), the residual xylanase activity was determined as described above.

PAGE and Molecular Mass Determination

For the determination of subunit molecular mass, sodium dodecylsulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed as described by Laemmli [20] with 10% gels. Protein bands were visualized with Coomassie brilliant blue R-250 (Sigma). The molecular mass of the purified enzyme was determined by size-exclusion chromatography using a Superose 12 (Amersham Pharmacia Biotech, Uppsala, Sweden) column attached to a ÄKTA FPLC system (GE Health Care). The enzyme was eluted with 20 mM sodium acetate (pH 5.0) at a flow rate of 0.5 ml/min.

Substrate Specificity

Substrate specificity of the purified enzyme was investigated in the standard assay system containing equal concentrations [1% (w/v)] of the following substrates: oatspelt xylan, birchwood xylan, beechwood xylan, Avicel, carboxylmethyl cellulose, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-galactopyranoside, *p*-nitrophenyl-β-D-lactopyranoside, *p*-nitrophenyl-α-L-arabinofuranoside.

Determination of Kinetic Parameters and Inhibition Constants

The values of the Michaelis constant (K_m) and the maximum velocity (V_{max}) were determined for xylanase by incubating in 100 mM sodium acetate buffer (pH 5) at 50°C with oatspelt xylan at concentrations

ranging from 0.2 to 5 mg/ml. Inhibition of xylanase by xylose was determined in the presence of oatspelt xylan as the substrate. Values for K_m and V_{max} and K_i were determined from Lineweaver–Burk plots using standard linear regression techniques.

Effects of Metals and Reagents

The effects of various metal ions and reagents at 1 mM on xylanase activity were determined by preincubating the enzyme with the individual reagents in 20 mM sodium acetate buffer (pH 5.0) at 30°C for 30 min. Activities were then measured at 50°C for 15 min in the presence of the metal ions or reagents. The activity assayed in the absence of metal ions or reagents was recorded as 100%.

Internal Amino Acid Sequence of Xylanase

The purified protein was resolved by SDS–PAGE and then electroblotted onto a polyvinylidene trifluoride membrane (Bio-Rad). Protein cleavage for peptide mapping was carried out at 37°C for 4 h with 100 ng of endoproteinase Asp-N or endoproteinase Lys-C or trypsin (Promega, Madison, WI, U.S.A.) to digest 20 μg of purified enzyme in 50 μl of 100 mM $(NH_4)_2CO_3$ (pH 8.5). The resulting peptide fragments were separated by SDS–PAGE (15% polyacrylamide), and the separated peptides were transferred to a polyvinylidene trifluoride membrane by electroblotting. Peptide bands were visualized by 0.1% Coomassie brilliant blue R-250 staining in 40% methanol. The partial amino acid sequence was determined by Edman degradation with an automatic protein sequencer (model 491A; Applied Biosystems, Division of Perkin-Elmer) at The National Instrumentation Center for Environmental Management (Seoul, Korea). The partial amino acid sequence was used to identify analogous proteins through a BLAST search of the nonredundant protein database.

Table 1. Effects of various carbon and nitrogen sources on xylanase production.

Carbon source (20 g/l)	Xylanase activity (U/mg-protein)	Total protein (mg)	pH
Cellulose	4.0±0.27	4.7±0.22	3.92
Glucose	1.7±0.11	2.1±0.11	2.57
Lactose	1.5±0.13	4.8±0.23	2.95
Maltose	2.1±0.25	2.9±0.24	2.72
Cellobiose	1.2±0.11	3.3±0.22	2.63
CMC	3.6±0.21	3.7±0.18	3.90
Xylan	4.8±0.47	4.9±0.11	3.99
Rice straw	5.1±0.52	5.2±0.13	3.71
Avicel	4.3±0.30	6.5±0.22	3.83
Sucrose	2.4±0.21	4.5±0.31	3.36
Softwood powder	1.2±0.11	4.0±0.21	5.67
Hardwood powder	3.1±0.22	8.6±0.42	3.77
Nitrogen sources (10 g/l)	**Xylanase activity (U/mg-protein)**	**Total protein (mg)**	**pH**
Yeast extract+peptone	5.4±0.32	5.1±0.23	3.92
Yeast extract	5.1±0.52	5.2±0.30	3.71
Peptone	3.3±0.23	3.7±0.15	3.95
Corn steep powder	4.5±0.33	2.5±0.17	4.01
Urea	2.6±0.11	2.9±0.04	7.48
$(NH_4)_2SO_4$	1.8±0.08	1.1±0.14	5.05
KNO_3	4.1±0.15	0.7±0.02	4.86
$NaNO_3$	4.7±0.02	1.0±0.22	3.37

All flasks were incubated at 25°C and 150 rpm for 15 days. Each value represents the mean of triplicate measurements and varied from the mean by not more than 15%.

RESULTS

Screening and Selection of Xylanase-Producing Strain

Among 112 fungal strains screened for xylanase activity, 15 strains were selected based on the zone of clearance observed in the agar plates containing 0.4% of Remazol Brilliant Blue xylan. Out of 15 strains, an efficient xylanase-producing *Fomitopsis pinicola* KMJ812 was selected for further study.

Optimization of Carbon and Nitrogen Sources for Xylanase Production

To select a suitable carbon source for xylanase production, *F. pinicola* KMJ812 was cultivated in media with yeast extract (10 g/l) and varied carbon sources (cellulose, rice straw, wheat bran, xylan, Avicel, CMC, cellobiose, glucose, maltose, lactose, sucrose, or wood fiber). Among the carbon sources tested, rice straw was found to be the best carbon source for xylanase production, leading to a xylanase-specific activity of 5.1 U/mg-protein in a flask culture (Table 1).

Since the mechanisms that govern the formation of extracellular enzymes are influenced by the availability of precursors for protein synthesis, the effects of inorganic and organic nitrogen sources on xylanase synthesis were also studied (Table 1). Rice straw (20 g/l) was used as a carbon source. Among the various nitrogen sources (peptone, corn steep powder, yeast extract, urea, ammonium sulfate, potassium nitrate, and sodium nitrate), a combination of yeast extract (5 g/l) and peptone (5 g/l) favored maximum xylanase production (5.4 U/mg-protein), followed by yeast extract, whereas the other nitrogen sources were poor sources of nitrogen in the absence of pH control. The time course of xylanase production by *F. pinicola* grown on rice straw, yeast extract, and peptone was studied (Fig. 1). Xylanase activity increased up to 17 days, after which it decreased gradually.

Purification of a Xylanase

Xylanase was purified as described in the Materials and Methods section, and the results are summarized in Table 2. Fractionation by ultrafiltration increased the specific activity about 2-fold, with 13% recovery of xylanase activity. The active fractions were applied to a DEAE-Sepharose column, and xylanase was eluted with approximately 0.1 M NaCl. A subsequent gel filtration step produced three peaks containing protein; the second peak showed xylanase activity.

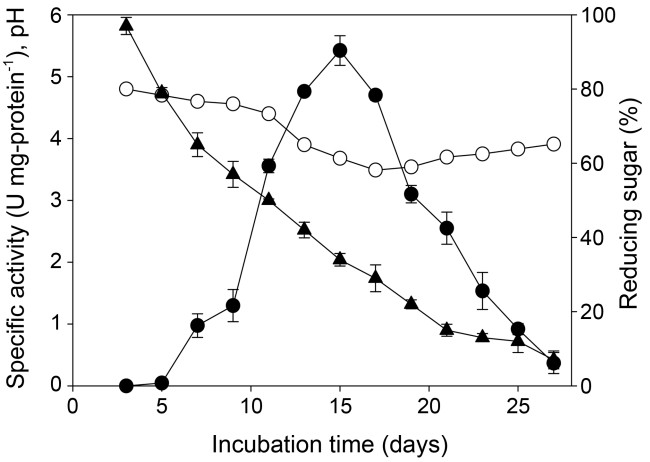

Fig. 1. Time course of xylanase production by *F. pinicola* KMJ812 on rice straw.
Filled circles, specific activity; empty circles, pH; filled triangles, residual reducing sugar level.

FPLC elution of a MonoQ ion-exchange chromatography column with 0.5 M NaCl produced an active xylanase protein peak. These chromatography methods resulted in a 14-fold purification of xylanase with a recovery of 0.1%. Analysis of the purified enzyme by gel electrophoresis in the presence of SDS (Fig. 2A, lane4) revealed one band with a M_r of 58,000. Size-exclusion chromatography on a Sephacryl S-300 high-resolution column resulted in the elution of the enzyme activity as a symmetrical peak corresponding to a M_r of approximately 58,000 (Fig. 2B). These results indicate that the enzyme migrates as a monomer in gel filtration under the mild conditions used and, thus, may also be present and active as a monomer in solution.

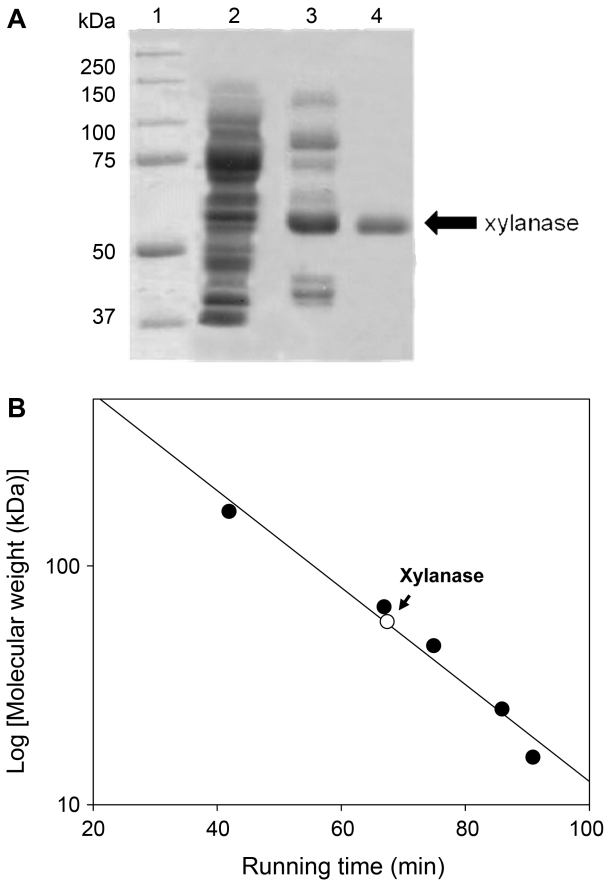

Fig. 2. PAGE and determination of molecular mass of xylanase purified from the *F. pinicola*.
A. PAGE of xylanase purified from *F. pinicola*. Lane 1, molecular mass marker; lane 2, cell extract; lane 3, DEAE ion-exchange fraction; lane 4, SDS–PAGE of MonoQ ion-exchange fraction. **B**. Determination of native molecular mass of *F. pinicola* xylanase by gel filtration chromatography on a Sephacryl S-300 high-resolution column. The column was calibrated with standard molecular mass proteins such as aldose (168 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa).

Identification of the Partial Peptide Fragment

The pure enzyme (1.5 μg) was separated by 10% SDS–PAGE and blotted onto a polyvinylidene fluoride (PVDF) membrane. Automated Edman degradation of the enzyme protein was unsuccessful, implying that the N-terminal of the enzyme was blocked. The xylanase was partially digested with trypsin, endoproteinase Asp-N, and endoproteinase Lys-C, and then separated by 12.5% SDS–PAGE and blotted onto a PVDF membrane. Three fragments [a Lys-C fragment (LYS), an Asp-N fragment (ASP), and a trypsin fragment (TRY)] were sequenced on an automatic protein sequencer. The LYS fragment contained a YKEFFKIGAAVTVK segment. The TRY and ASP fragments contained LSDVEEAIRIVR and NNESLICTNSDGTLD, respectively.

Table 2. Purification of xylanase from the culture broth of *F. pinicola* KMJ812.

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg-protein)	Yield (%)	Purification fold
Cell extract	1,370	1,150	5.4	100	1
Ultrafiltration (PES30)	177	210	11.8	13	2.2
Salting out	56	107	19.1	4.1	3.5
DEAE ion-exchange chromatography	37	94	25.6	2.7	4.7
Gel filtration chromatography	11	63	55.4	0.8	10.3
MonoQ ion-exchange chromatography	1	8	74.4	0.1	13.8

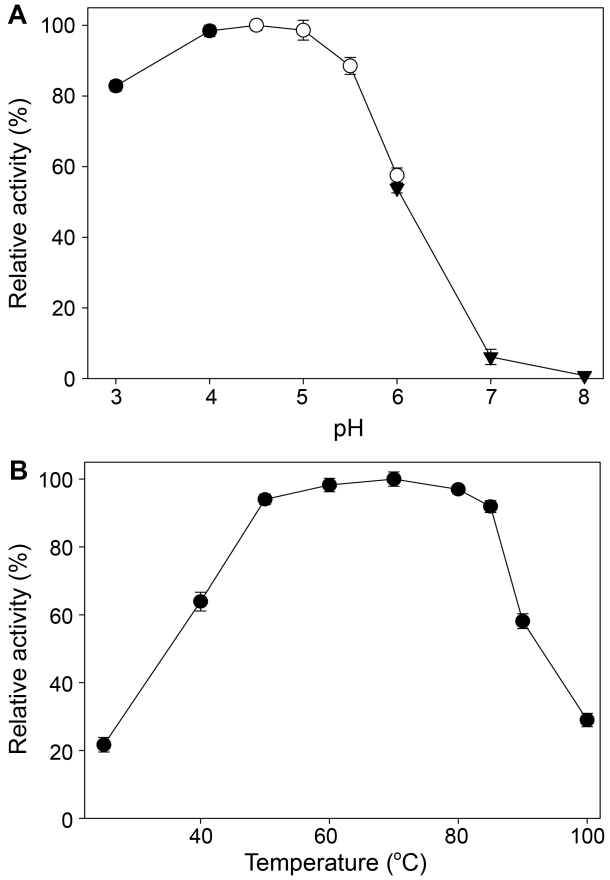

Fig. 3. Effects of pH and temperature on the activity of *F. pinicola* xylanase.
A. Effect of pH on the activity of purified xylanase from *F. pinicola* KMJ812. The enzyme activity was assayed by the standard assay method by changing the buffer to obtain the desired pHs. The buffers used were citrate (pH 3.0 to 4.5), sodium acetate (pH 4.5 to 6.0), and phosphate (pH 6.0 to 8.0). **B**. Effect of temperature on the activity of purified xylanase from *F. pinicola* KMJ812. The enzyme activity was assayed at various temperatures by the standard assay method. Each value represents the mean of triplicate measurements and varied from the mean by not more than 15%.

Optimum pH and Temperature

The optimum pH for the xylanase was 4.5, with 98% and 98% of the maximum activity appearing at pH 4.0 and 5.0, respectively (Fig. 3A). An acidic pH optimum and maximal activity at about pH 4.5 are common features of similar xylanase enzymes isolated from diverse microbial systems [33]. The isoelectric point, pI, was determined to be 4.7, which is typical for extracellular xylanases [1, 6]. The optimum temperature for the hydrolysis reaction was 70°C with 94% and 96% of the maximum activity at 50°C and 80°C, respectively (Fig. 3B). It still retained approximately 50% relative activity at 90°C.

Thermostability of *F. pinicola* Xylanase

The stability of purified xylanase was studied at various temperatures from 40°C to 80°C. The purified *F. pinicola* xylanase was highly stable and maintained ~100% activity when incubated at 40°C for 25 days. About 90% activity was maintained after 60 h incubation at 60°C. At temperatures over 80°C, the xylanase activity sharply decreased depending on the incubation time. The enzyme showed $t_{1/2}$ values of 410 h, 96 h, and 33 h at 50°C, 60°C, and 70°C, respectively (Fig. 4).

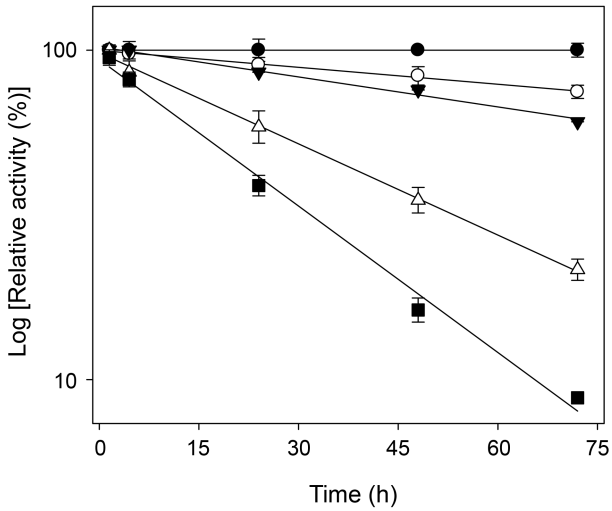

Fig. 4. Thermal inactivation of *F. pinicola* xylanase.
The enzymes were incubated at 40°C (filled circles), 50°C (empty circles), 60°C (filled triangles), 65°C (empty triangles), and 70°C (filled squares) for varying periods of time. Samples were withdrawn at each time interval and relative activities were determined.

Substrate Specificity and Kinetic Parameters of *F. pinicola* Xylanase

The activities of *F. pinicola* xylanase with various substrates are shown in Table 3. The highest activity (105%) was observed with the birchwood xylan, followed by the oatspelt xylan (100%). The enzyme exhibited low activities towards celluosic substrates, such as Avicel (11%) and CMC (7.2%).

Initial velocities were determined in the standard assay mixture at pH 5. All of the substrates tested had hyperbolic saturation curves, and the corresponding double-reciprocal plots were linear. The Lineweaver–Burk plot (Fig. 5) obtained for the conversion of oatspelt xylan under standard assay conditions showed a V_{max} of 80.2 U/mg-protein, K_m of 3.4 mg/ml, and k_{cat} of 77.4 s^{-1}. Under the nonsaturating conditions, xylose inhibition was competitive for oatspelt xylan (Fig. 6), and xylose bound to *F. pinicola* xylanase with a K_i value of 13.4 mM.

Effects of Metal Ions and Various Compounds

Xylanase activity was assayed in the presence and absence of metal ions, a metal chelator (EDTA), and various other compounds (Table 4). Xylanase activity was slightly activated by $BaCl_2$ and NaCl. $CuCl_2$, $HgCl_2$, and $MnCl_2$ strongly inhibited the activity, whereas $CaCl_2$, $CoCl_2$, $FeCl_2$, $MgCl_2$,

Table 3. Substrate specificity of xylanase purified from *F. pinicola* KMJ812.

Substrate	Xylanase activity (U/mg-protein)	Relative activity (%)
Oatspelt xylan	74.4	100±9.1
Birchwood xylan	78.1	105±8.2
Beechwood xylan	57.3	77±6.6
Avicel	8.2	11±1.0
Carboxylmethyl cellulose	5.4	7.2±0.7
p-Nitrophenyl-β-D-xylopyranoside	0.02	0
p-Nitrophenyl-α-L-arabinofuranoside	0.001	0
p-Nitrophenyl-β-D-cellobioside	0.1	0
p-Nitrophenyl-β-D-lactopyranoside	0.01	0
p-Nitrophenyl-β-D-galactopyranoside	0	0

The activity for oatspelt xylan was defined as 100%. The purified enzyme was assayed in the standard assay condition with various compounds. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%.

and $ZnCl_2$ had much more limited inhibitory effects. The enzyme was inhibited by EDTA at concentrations ranging from 1 to 10 mM.

The effects of sulfhydryl compounds on the xylanase from *F. pinicola* were also examined (Table 4). The addition of 10 mM 2-mercaptoethanol and cysteine to the reaction mixture increased the enzyme activity by 135.3% and 119.3%, respectively. These results suggest that sulfhydryl compounds keep the active enzyme in a reduced state. This may merely indicate that xylanase contains easily accessible disulfide bonds that are readily reduced.

Discussion

Although the purification and properties of xylanase from several strains have been reported, this is the first report on the purification and characterization of xylanase from *F. pinicola*, a basidomycete. The extracellular xylanase purified from *F. pinicola* was a monomer with a molecular mass of

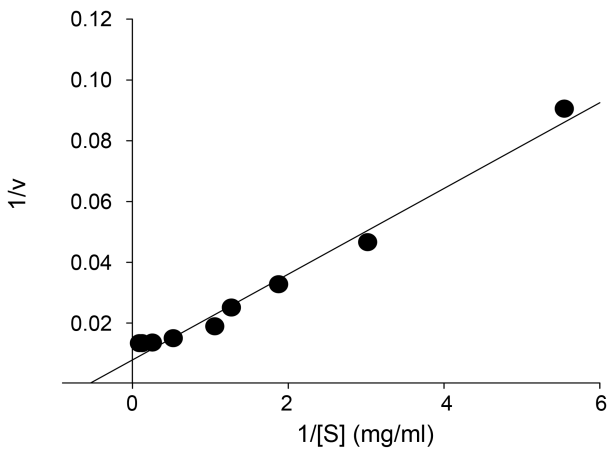

Fig. 5. Lineweaver–Burk plot of initial velocity versus various fixed substrate concentrations.
Xylanase activity was measured in the presence of the indicated concentrations of oatspelt xylan at pH 5.0. Each value represents the mean of triplicate measurements and varied from the mean by not more than 15%.

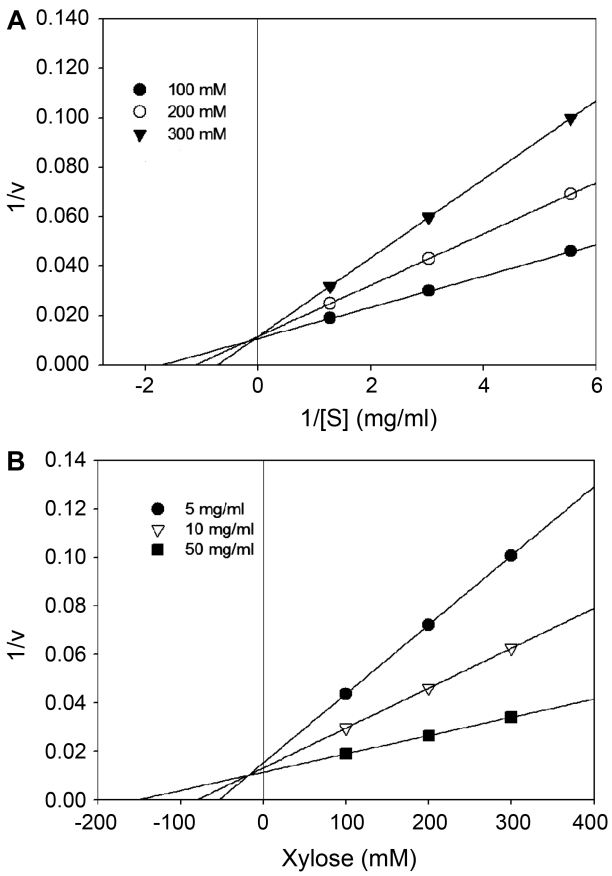

Fig. 6. Graphical analysis of the inhibition of *F. pinicola* xylanase by xylose.
A. Lineweaver–Burk plot of initial velocity versus various fixed substrate concentrations showing inhibitory effects of xylose on oatspelt xylan hydrolysis by *F. pinicola* xylanase. **B**. The secondary plot for competitive inhibition with oatspelt xylan is shown. The xylose product binds to xylanase with a K_i of 13.4 mM.

Table 4. Effects of metal ions and chemical reagents on the activity of xylanase purified from *F. pinicola* KMJ812.

	Concentration (mM)	Specific activity (U/mg-protein)	Relative activity (%)
None	–	74.4	100±9.6
EDTA	10	63.5	85.4±4.9
$BaCl_2$	10	74.3	99.8±8.2
NaCl	10	74.6	100±4.6
$CaCl_2$	10	83.6	112±4.4
$CoCl_2$	10	97.2	131±5.6
$CuCl_2$	10	44.4	59.7±2.1
$FeCl_2$	10	105	142±9.7
$HgCl_2$	10	19.8	26.6±1.4
$MgCl_2$	10	91.0	122±6.4
$MnCl_2$	10	31.2	42.0±2.1
$ZnCl_2$	10	109	147±5.6
β-Mercaptoethanol	10	101	135±7.8
L-Cysteine	10	88.8	119±11.5

Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%.

58 kDa. The molecular mass of *F. pinicola* xylanase is in agreement with those of many extracellular xylanases characterized from other fungal sources [12, 32]. In general, GHs including xylanases are characterized by relatively broad substrate specificities [7, 11]. *F. pinicola* xylanase also shows relatively broad substrate specificities (Table 3).

Table 5 shows a comparison of the properties of various xylanases from a number of different sources. *F. pinicola* xylanase had a comparable specific activity of 74.4 U/mg-protein for oatspelt xylan. In comparison, specific activity values of purified xylanases from other fungi range from 7.4 to 894 U/mg-protein (Table 5). Depending on the optimal temperature, enzymes can be classified as mesophilic (40–60°C), thermophilic (50–80°C), and hyperthermophilic (>80°C) [29]. In recent years, a lot of effort has been put into the isolation of thermophilic and even extremophilic microorganisms, since they produce enzymes of greater stability [5, 24]. The xylanases from thermophilic strains like *Talaromyces emersonii*, *Thermomyces lanuginosus*, and *Thermoascus aurantiacus* possess optimum temperatures between 60°C and 80°C and are very stable in this range [29]. Many endoxylanases from thermophiles have some degree of structural homology with those from mesophiles. The xylanase from *F. pinicola*, a mesophile, showed a significant stability at high temperatures. The enzyme showed the highest thermostability with a half-life of 33 h at 70°C (Table 5).

Family 10 xylanase is known to have the $(\beta/\alpha)_8$ barrel structure and belongs to the so-called 4/7 superfamily of GHs [17]. The catalytic domain structures of native and

Table 5. Properties of xylanases from various sources.

Microorganism	M_r (kDa)	Quaternary structure	Opt. pH	Opt. temp (°C)	Specific activity (U/mg-protein)	Half-life ($t_{1/2}$)	Reference
Laetiporus sulphureus	69.3	Monomer	3	80	72.4	4 h at 60°C	[22]
Paecilomyces thermophila	53.5	Monomer	6.5	55	43.4	1 h at 60°C	[32]
H. grisea var. *thermoidea*	29	Monomer	4.5–6.5	55–60	10.2	5.5 h at 60°C	[23]
*Lentinus edodes*M290	35	Monomer	4	50	90.4	4 h at 60°C	[21]
Penicillium citrinum	25	Monomer	6.0	50	69.6	NR	[30]
Streptomyces olivaceoviridis E-86	45	Monomer	NR	NR	26.5	NR	[16]
Streptomyces cyaneus SN32	20.5	Monomer	6.0	60–65	894	0.8 h at 65°C	[27]
Penicillium janthinellum	NR	NR	4.5	50	84.4	1.8 min at 60°C	[9]
Aspergillus aculeatus	52	Monomer	5.0	50	10.1	0.5 h at 50°C	[12]
Termitomyces sp.	80	Monomer	5.6	65–70	26.4	3 h at 60°C	[11]
Chaetomium cellulolyticum	57	Monomer	7.0	50	11	3 h at 50°C	[2]
Sporotrichum thermophile	45	Monomer	7.0	50	83.2	1 h at 60°C	[18]
Fomitopsis pinicola	58	Monomer	4.5	50	74.4	33 h at 70°C	This work

NR, not reported.
Kinetic parameters of xylanases were shown for xylan.

complex forms of family 10 xylanases are available from several microorganisms including *Pseudomonas fluorescens* [28], *Streptomyces olivaceoviridis* [13], and *Geobacillus stearothermophilus* [35]. The internal amino acid sequence analysis of *F. pinicola* xylanase showed similarity towards GH family 10 xylanases. The fragments of *F. pinicola* xylanase, YKEFFKIGAAVTVK, SDVEEAI, and ESLICTNSDGTLD, are identical to those of the xylanases from *Anaerocellum thermophilum, Bacteroides fragilis*, and *Sphingobacterium spiritivorum*, respectively. The evidence from enzymology and bioinformatics experiments suggests that *F. pinicola* xylanase should be classified as a member of GH family 10.

In conclusion, a highly thermostable xylanase was purified and characterized from *F. pinicola* KMJ812. The successful purification and characterization of xylanase produced by *F. pinicola* allows us to characterize a novel xylanase showing high thermostability and now sets the stage for more detailed investigations of this novel GH, such as X-ray crystallography and the cloning of the full-length gene followed by protein engineering studies.

Acknowledgment

This study was supported by a grant (S210707L010120) from the Korea Forest Service.

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