Purification, Characterization, and Molecular Cloning of Acidophilic Xylanase from *Penicillium* sp.40

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*Penicillium* sp. 40, which can grow in an extremely acidic medium at pH 2.0 was screened from an acidic soil. This fungus produces xylanases when grown in a medium containing xylan as a sole carbon source. A major xylanase was purified from the culture supernatant of *Penicillium* sp. 40 and designated XynA. The molecular mass of XynA was estimated to be 25,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. XynA has an optimum pH at 2.0 and is stable in pH 2.0–5.0. Western blot analysis using anti-XynA antibody showed that XynA was induced by xylan and repressed by glucose. Also, its production was increased by an acid medium. The gene encoding XynA (xynA) was isolated from the genomic library of *Penicillium* sp. 40. The structural part of xynA was found to be 721 bp. The nucleotide sequence of cDNA amplified by RT-PCR showed that the open reading frame of xynA was interrupted by a single intron which was 58 bp in size and encoded 221 amino acids. Direct N-terminal amino acid sequencing showed that the precursor of XynA had a signal peptide composed of 31 amino acids. The molecular mass calculated from the deduced amino acid sequence of XynA is 20,713. This is lower than that estimated by gel electrophoresis, suggesting that XynA is a glycoprotein. The predicted amino acid sequence of XynA has strong similarity to other family11 xylanases from fungi.

Key words: acidophilic; *Penicillium*; xylanase

Hemicellulose is the second most abundant group of polysaccharides of plant cell walls. It is composed of xylans, arabinans, galactans, glucans, and mannans. Among these polysaccharides, xylan is one of the major hemicellulosic components of wood and agricultural residues. Endo-β-d-1,4-xylanase (EC.3.2.1.8) which attacks the polysaccharide backbone of d-xylans is the most important enzyme to degrade xylan. The hydrolysis of xylan is of great interest for various biotechnological applications.¹⁻³ Commercially available xylanases are usually produced by filamentous fungi such as *Aspergillus niger* and *Trichoderma reesei*. These xylanases are used in the pulp and paper industry to bleach pulp and also used as a supplement in chicken feed for effective digestion.

*Penicillium* sp. 40 was isolated from a hot spring. This fungus can grow in acidic conditions such as pH 2.0. When grown on xylan as the only carbon source in the acidic medium, this fungus produces a complete set of enzymes capable to degrade xylan. These enzymes are acid stable and have negligible activity towards cellulose. Because of this cellulase-free activity and extremely low pH optimum, these xylanases are useful for bleaching kraft pulp. To date, several acid-stable xylanases from fungi have been reported.⁴⁻⁷ Recently, X-ray crystallography of acidophilic xylanase C from *Aspergillus kawachii* and mutationanalyses were done.⁸ Several structural features responsible for acid stability were demonstrated. In this paper, we describe the purification, characterization, and gene cloning of a unique acid-stable and acidophilic xylanase from this *Penicillium* strain and compared it with other acid-stable xylanases to identify the structural basis of its acid stability and low pH optimum.

Materials and Methods

Organism and culture conditions. *Penicillium* sp.40 was isolated from the Zaoh hot spring, Yamagata, Japan during a screening program for acidophilic fungi. The organism was stored on potato/dextrose/agar slants at 4°C. For enzyme production, the basic medium (0.5% polypeptone, 0.1% yeast extract, 0.01% potassium-phosphate, 2% glucose, 0.2% β-methyl-d-xylloside (βMX), pH was adjusted to 1.8 by hydroxy-chloride) was used. The 500-ml flasks containing 200 ml of medium were inoculated with the conidiospores. Incubation was

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Abbreviations: βMX, β-methyl-d-xylloside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
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done on an orbital shaker at 25°C, 130 rpm for 10 days. The *E. coli* strain used for recombinant DNA methods was DH5α.

**Identification of fungus.** Classification of this fungus was done based on cultural and morphological characteristics of colonies described elsewhere. The colony of this fungus grew well on Czapek’s solution agar. The colony of the fungus was blue-green after the full growth on the basic medium. Morphological features of the fruiting structure was observed with a microscope. The aerial mycelium of this fungus was colorless. The structure of the conidiophore was typical of the genus *Penicillium*. The branched part of the conidiophore above the stipe had the penicillus structure. Based on these observations, we identified the fungus as a *Penicillium* species.

**Biochemical assays.** Xylanase activity was measured by incubating for 10 min at 25°C, a 0.2-ml reaction mixture containing appropriately diluted enzyme in 50 mM citrate buffer, pH 2.0, and 7.5 mg/ml oatspelt xylan (Fluka Ag). The reducing sugars produced were assayed by the 3,5-dinitrosalicylic acid method using xylose as standard. One unit of activity was defined as the amount of enzyme producing 1 μmol product equivalents per min. Protein was measured by the Bradford protein assay kit (Bio-rad Laboratories) using bovine serum albumin fraction V (Sigma) as the standard.

**Electrophoresis of proteins.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done in 12% acrylamide gel as described by Laemmli. The gels were stained with Coomassie Brilliant Blue R 250. Activity staining of xylanase after SDS-PAGE was done by the method described previously. Isoelectric focusing was done on polyacrylamide gels containing Bio-Lyte 3/10 (Bio-Rad Laboratories) ampholyte in the pH range 3.7-9.1 by comparison with protein standards of known pI. Protein bands were stained with Coomassie Brilliant Blue G 250.

**Western blot analysis.** Proteins in one ml of each culture supernatant were pelleted with trichloroacetic acid and run on SDS-PAGE. After the electrophoresis, separated proteins were electrophorotated onto the Nylon membrane (ADVANTEC) and horse-radish peroxidase linked to anti-IgG antibody (Tago Inc.) was used to detect the antigen-antibody complexes.

**Xylanase purification.** A one-liter culture was filtered, and proteins were precipitated with ammonium sulfate at 70% saturation. The precipitate was dissolved in 10 mM phosphate buffer (pH 7.0) and dialyzed against the same buffer. The resultant solution was put onto a DEAE-Toyopearl 650 M column (2.5 × 20 cm) (Tosoh) equilibrated with the same buffer. Elution was done with a linear NaCl concentration gradient (0-0.5 M in the same buffer). Xylanase fractions were pooled, dialyzed against 10 mM potassium-phosphate buffer (pH 5.5), concentrated by membrane filtration, and then put on a Superdex 200 pg column (1.6 × 60 cm)(Pharmacia). Active fractions were combined and used as the purified enzyme. The characterization and substrate-hydrolysis pattern described in this study were done with the purified enzyme.

**N-terminal amino acid sequencing of XynA.** Five micrograms of XynA was treated with pyrogulatinase (Takara Shuzo) as the manufacturer’s protocol directed and then run on SDS-PAGE. After electrophoresis, protein was electrophorotated onto a PVDF membrane (Millipore). The protein was stained with Coomassie Brilliant Blue R250 and was cut off from the membrane. Its N-terminal amino acid sequence was analyzed on an Applied Biosystems model 476A protein sequencer.

**Cloning of the xynA gene.** To amplify the xylanase gene using the polymerase chain reaction (PCR), two oligonucleotide primers, xynA-F: 5’-GA(AG)TA(TC)TA(TC)AT(TCA)GT(ATGC)GA(AG)(GA)A-3’ and xynA-R: 5’-GCCCA(AG)AA(AG)T(TT)(AG)-AA(AG)TG(AG)TT-3’ were synthesized based on the amino acid sequences, which are highly conserved among fungal xylanases. The chromosomal DNA from *Penicillium* sp.40 was isolated as described elsewhere and PCR was done for 30 cycles according to the following cycle profile: 60s at 94°C, 60s at 45°C, 60s at 72°C, using ampli-Taq polymerase (Perkin Elmer). The resulting fragment gave a discrete band of about 220 bp and was cloned to pUC119 and five independent inserts were sequenced. They comprised only one sequence and encoded 75 amino acids, the sequence of which was highly similar to those of other fungal xylanases. Before the construction of the partial genomic library to isolate the complete gene, genomic Southern hybridization was done using this PCR fragment as a probe under standard conditions to identify specific restriction fragments of interest. The fragment was labeled with digoxigenin-11-DUTP (Boehringer Manheim Biochemica) and used to screen the genomic library. A hybridized band appeared in the DNA fragments digested with EcoRI (5.5 kb), *XbaI* (1.8 kb) and *XhoI* (0.8 kb). The *Penicillium* sp.40 EcoRI gene library was constructed using the pBluescript II KS (+) vector (Stratagene). Screening of this library with the PCR fragment as a probe resulted in 5 positive clones. Restriction analysis of these clones indicated that they harbored the same insert. A 1.8 kb *XbaI* fragment in this insert was subcloned into pUC119 and subjected to sequencing.
Table 1. Purification of Xylanase from *Penicillium* sp.40

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification fold (fold)</th>
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<td>23200</td>
<td>462</td>
<td>50.3</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>13800</td>
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<td>245</td>
<td>59.0</td>
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<tr>
<td>DEAE-Toyopearl</td>
<td>9890</td>
<td>12.6</td>
<td>785</td>
<td>42.6</td>
<td>15.6</td>
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<tr>
<td>Superdex 200 pg</td>
<td>7150</td>
<td>5.71</td>
<td>1250</td>
<td>30.8</td>
<td>25.9</td>
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</tbody>
</table>

**DNA sequencing.** The sequence was analyzed by a Licor model 4000L automated DNA sequencer using a Thermo Sequenase cycle sequencing kit (Amersham Life Science Inc). The nucleotide sequence data was analyzed with GENETYX computer software (Software Development Co. Ltd.). Sequence similarity searches in GenBank were done with a BLAST program.

**Other methods.** All of the DNA manipulations were done using standard methods as described by Sambrook *et al.*

**Nucleotide sequence accession number.** The sequence has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number AB035540.

**Results and Discussion**

**Purification and characterization of XynA**

The yield of each step of the enzyme purification procedure is presented in Table 1. During purification, enzyme activity increased 25.9 times, giving 30.8% yield. SDS-PAGE of purified enzyme indicated a single protein with a molecular weight of 25,000 (Fig. 1). The isoelectric point of XynA was 4.7. Enzymatic properties of XynA are summarized in Table 2. The optimum pH of XynA was 2.0 and it retained over 95% of its maximal activity in the pH range from 2.0 to 5.0. Even at pH 1.0, more than 70% of the maximum activity was retained. Also, the enzyme retained 60% of its activity in pH 8.0. Therefore, XynA was an acidophilic enzyme. The temperature optimum of XynA was 50°C. The purified enzyme showed complete stability at 30°C up to 10 min incubation in pH 2.0 solution. The optimum temperature of XynA was similar to that of xylanases from many mesophilic fungi. However, the temperature stability of XynA was lower than that of xylanases from other fungi. The influence of divalent metal cations on xylanase activity at 8 mM was examined: Mg^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, or Ni^{2+} showed no or slight inhibitory effects, whereas, addition of Mn^{2+}, Fe^{2+} and Cu^{2+} partly inhibited the enzyme. Strong inhibition was produced by Hg^{2+}. Effects of these cations on XynA activity were similar to those reported for other xylanases. The purified XynA had typical Michaelis-Menten kinetics. The Km of 8.3 mg/ml was similar to those described for other fungal acid-stable xylanases. However, the V_max of 6100 U/mg was higher than other fungal xylanases. Hydrolytic activity of XynA to different substrates was examined by incubation at pH 2.0 and 25°C. The purified enzyme showed high activity toward xylan. XynA had no significant activity against carboxyl-methyl-cellulose, Avicel, p-nitrophenyl-xyloloside, or xylobiose. The products of a hydrolysis of xylan by XynA were analyzed by thin-layer chromatography. The reaction products contained xylo-oligosaccharides and the
main product was xylobiose. Since no β-xylosidase activity was detected, these results confirm that XynA is an endoxylanase. The results from enzyme characterization indicated that XynA belongs to low molecular weight endoxylanases, which are classified as family 11 glycosidases.39

The most striking feature of XynA was its pH stability and optimum pH. In general, xylanases of fungal origin have been shown to be acid-stable because fungi grow well in acidic conditions around pH 4-5. However, Penicillium sp. 40 can grow well in pH 2.0 and even in pH 1.0, it can still grow. Several xylanases with low pH optimum have been reported.4-7,16,17 Among them, Xylanase C from A. kawachii39 and Xylanase CS from Cryptococcus sp. S-240 had extremely low pH optima. The acid-stable and acidophilic features of XynA were comparable to those xylanases.

**Xylanase production in Penicillium sp. 40**

Inductive effects of various saccharides on xylanase production by Penicillium sp. 40 were examined (Fig. 2). In filamentous fungi, non-metabolized βMX is known to be the most potent inducer of xylanases.4-22 To examine whether βMX could be used for the xylanase production as an available inducer in Penicillium sp. 40, various saccharides were added to the culture medium after glucose in the medium was consumed. In Penicillium sp. 40, the production of xylanase induced by βMX was greater than that by xylan and xylo-oligosaccharide. The xylanase induction was dependent on the concentration of βMX and the most effective induction was obtained at 1 mg/ml of βMX. Since βMX was slowly hydrolyzed and metabolized by Penicillium sp. 40, xylanase induction by βMX continued for a long time and high level of xylanase activity was produced. Xylanase production was completely inhibited in the presence of glucose. Penicillium sp. 40 effectively produced xylanase activity in the acid medium (pH 2.0) containing xylan as a carbon source. Production of xylanase activity was reduced in weakly acidic media such as pH 5.0. Also, the growth in pH 5.0 was slightly slower than that in pH 2.0.

To examine the enzymes produced in the culture supernatant of each medium, proteins in that fraction were analyzed by SDS-PAGE and Western blot analysis. In the culture supernatant of xylan medium at pH 2.0, only one major protein band with a molecular mass of 25,000 was stained by Coomassie Brilliant Blue, and anti-XynA antibody cross-reacted with this protein by Western blot analysis. In pH 5.0 medium, the XynA content was smaller than that in pH 2.0 medium. The culture supernatant of the glucose medium did not contain any visible protein bands. These findings suggest that XynA production was subjected to catabolite repression by glucose and pH regulation. A. kawachii has been reported to produce acidophilic Xylanase C in xylan and low pH dependent manner.39 Induction conditions of XynA by Penicillium sp. 40 were similar to that of Xylanase C by A. kawachii.

**Cloning and nucleotide sequencing of the xynA gene**

Since purification of XynA and its characterization suggested that XynA belongs to the family 11 xylanase, two oligonucleotide primers were synthesized based on the conserved amino acid sequences among family 11 xylanases and a part of the xynA gene was amplified. A partial genomic library of Penicillium sp.40 constructed in pBluescript II KS (+) was screened with the PCR-amplified DNA fragment as described in Materials and Methods. Of 5,000 colonies screened, 5 positive clones were obtained. A 1.8-kb XbaI fragment of the insert in these clones was hybridized to the probe on Southern blot analysis. This fragment was subcloned to pUC119. A detailed restriction fragment map was constructed (Fig. 3) and sequencing was done for both strands of the fragment (Fig. 4). Although the exact translation initiation site was not found, the structural part of xynA appeared to be 721 bp long and may be interrupted by a single intron of 58 bp. The exact position

![Fig. 2. Effects of Inducers and Courses of Extracellular Xylanase Production by Penicillium sp. 40.](image)

After 3 days of cultivation in glucose medium, each inducer was added to the medium and cultivation was done for 6 more days. ●, βMX (0.1%); ○, none; ▲, xylo-oligosaccharide (0.5%); ■, xylan (0.5%)

![Fig. 3. Restriction Map of the xynA Gene.](image)

Exons and introns are represented by black and open boxes, respectively.
of the intron was confirmed by sequencing the cDNA fragment amplified by RT-PCR. An intron/exon junction that followed the GT-AG rule was present and the size of the intron resembled those of other filamentous fungal introns. Moreover, the intron occurs at the same position as those of family 11 xylanases of filamentous fungi. The \textit{xynA} gene contained an open reading frame (ORF) encoding 221 amino-acid residues. Deducible amino acid sequence of the product of the ORF showed a perfect match to the direct N-terminal amino acid sequence of purified \textit{XynA}, indicating that \textit{xynA} encodes the \textit{XynA} protein and suggesting that the precursor of \textit{XynA} contains a signal sequence of 31 amino acids in its amino acid sequence.
Fig. 5. Alignment of the Amino Acid Sequences of the *Penicillium* sp. 40 XynA with Other Fungal Xylanases.

Sequences similar to all sequences are boxed in black. The putative catalytic Glu residues are marked by asterisks (*). Asp residues conserved in acidophilic xylanases are marked by #. Unique Cys residues are boxed in black and marked by @.
terminal. A mature enzyme is composed of 190 amino acids with a molecular weight of 20,713. This is lower than the molecular weight of XynA estimated by SDS-PAGE (25,000). This would be due to the glycosylation of XynA. In fact, PAS-staining of XynA showed that it is a glycosyl protein and the carbohydrate content of XynA measured by the phenolsulfate method was 114 mg sugar/g protein.

The 5'-noncoding region of the sequence was screened for various consensus sequences. Although no typical eukaryotic promoter with a TATA box (TATAAAAT) existed, a similar sequence, TATAAA was detected 89 bp upstream from the translation initiation site. The 5'-GGCTAAA-3'-like sequence identified as the binding site for XlnR, a transcriptional activator of the xylanolytic system in A. niger, was also observed at –164. Five sequences which are similar to the CREA-binding sites, 5'-G/CYGGGGG-3' as were identified for A. nidulans, were found within the region upstream of translation initiation site. These results suggest that similar transcrictional factors as XlnR and CreA participate in induction and repression of xynA in Penicillium sp. 40. Production of XynA depends on the pH of the culture medium. pH regulation of gene expression is known to be mediated by the transcriptional factor PacC. In A. nidulans, expression of the family I xylanase genes were shown to be regulated by PacC. In the 5'-upstream region, two sequences similar to the consensus sequence (5'-GCCARG-3') of PacC binding were identified, suggesting that expression of xynA in different pH medium would be regulated by a PacC-like factor. In common with some other fungal genes, the typical polyadenylation signal, AATAAA, was not present within the 3'-noncoding region.

Comparison of the amino acid sequence

The deduced amino acid sequence of XynA was compared to the published sequences for other fungal family I xylanases. The alignments of similar sequences of XynA and representative xylanases in family I1 are shown in Fig. 5. The amino acid identities of XynA to A. kawachii XynB (GenBank accession no. D38070), A. kawachii XynC, A. nidulans XlnA, A. nidulans XlnB, A. niger XynI, A. niger XynII, A. awamori ExI, Aureobasidium pullulans XynA, Cryptococcus sp. S-2 Xyn-C5, Trichoderma reesei XynI, and T. reesei XynII are 70%, 50%, 67%, 63%, 51%, 73%, 49%, 51%, 44%, 56%, and 61%, respectively. The putative catalytic residue, nucleophile Glu86 and catalyst Glu177 are identified in XynA. A crucial difference in enzymatic features among these xylanases was their optimal pHs. Penicillium sp. 40 XynA, A. kawachii XynC, A. niger XynI, A. awamori ExI, A. pullulans XynA, Cryptococcus sp. S-2 Xyn-C5 and T. reesei XynI had acidophilic features with an optimal pH from 2.0–4.0. In contrast, others have weak acidophilic features with an optimal pH of 4.5–5.5. The three-dimensional structures of acidophilic xylanase from A. niger, T. reesei, and A. kawachii have been reported and the importance of the Asp residues for their acidophilic feature were highlighted. Asp76, which are common in acidophilic xylanases, is also conserved in XynA. These residues are replaced by Asn in other fungal xylanases. It was proposed that the Asp residue interacts with the catalytic Glu residue by a hydrogen bond in higher pH and, only at low pH, the Asp residue can be protonated and the proton of the catalytic Glu is available for catalysis. The importance of this Asp residue in the acidophilic feature have been established by mutational analysis of XynC from A. kawachii. Our sequence result also supports the importance of this Asp residue in pH profiles of fungal acid stable xylanases. In XynC of A. kawachii, Cys91 was shown to form a disulfide bond with Cys110. Other acid stable xylanases with low pH optimum also have Cys residues at the same position, although it is not known how the disulfide bond contributes to the acid stability. Interestingly, Cys residues are not present at these positions in XynA from Penicillium sp. 40.

The primary structure of XynA was more similar to the primary structure of xylanases with neutral pH optimum than that with acid pH optimum. However, the Asp residue at the catalytic site of the enzyme was conserved in acidophilic xylanases. This suggests that acid stability or acidophilic feature of xylanases depends on the higher structure. Sequence comparison and mutational analyses of XynA might identify the mechanism underlying stability or acidophilic character of xylanases against low pH condition and enable us to engineer enzymes with different pH optima.

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