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Purification and Properties of an Extracellular Endo-1,4-β-Xylanase from *Penicillium citrinum* and Characterization of the Encoding Gene

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An extracellular endo-1,4-β-xylanase was purified from the culture filtrate of a filamentous fungus *Penicillium citrinum* FERM P-15944 grown on birch-wood xylan. The purified enzyme showed a single band on SDS–PAGE with an apparent M_r of 20,000 and had an isoelectric point below 3.5. Xylanase activity was optimal at pH 5.0 and 55°C. The genomic DNA and cDNAs encoding this protein were cloned and sequenced. Southern blot analysis indicated that the xylanase gene (*xynA*) was present as a single copy in the genome. An open reading frame of 657 bp was interrupted by two introns of 65 and 55 bp, and encoded a presumed prepropeptide of 27 amino acids and a mature protein of 190 amino acids. Three distinct transcription start points were observed at positions −20 (A), −31 (A), and −36 (A) from the start codon. The 5′-noncoding region had a putative TATA box at nt −66 (TATAAA). The *xynA* cDNA was functionally expressed under the control of the alcohol oxidase I gene promoter in the methylotrophic yeast *Pichia pastoris*. A neighbor-joining tree showed that the *P. citrinum* enzyme is closely related to several other fungal xylanases belonging to the glycoside hydrolase family 11: *Trichoderma reesei XYN2*, *Aspergillus niger xynNB*, *Penicillium funiculosum xynC*, *Penicillium* sp. strain 40 *xynA*, *Chaetomium gracile cgxB*, and *Aspergillus nidulans xlnA* and *xlnB*.

[**Key words:** extracellular enzyme, *Penicillium citrinum*, phylogenetic analysis, xylan, xylanase]

β-1,4-Xylan is a heterogeneous polysaccharide that has a backbone of β-1,4-linked D-xylopyranose residues commonly substituted by L-arabinofuranose, D-glucuronic acid, and 4-*O*-methylglucuronic acid. The xylan constitutes a major component of hemicellulose in the cell walls of monocots and hard woods (1). Xylanolytic enzymes have been the focus of much attention due to their potential applications in the pulp and paper, textile, and food industries and in agriculture (2). Endo-1,4-β-xylanases (EC 3.2.1.8), designated xylanases, hydrolyze the internal 1,4-β-D-xylosidic linkages of the xylan backbone to produce short-chain xylooligosaccharides (2). The majority of xylanases fall into glycoside hydrolase (GH) families 10 and 11 according to the similarities of amino acid sequences, whereas some other xylanases are classified into GH families 5, 8, and 43 (http://afmb.cnrs-mrs.fr/CAZY/). GH family 11 consists of xylanases with a relatively low M_r ranging from 19,000 to 25,000 and GH family 10 consists of xylanases with a high M_r of >30,000 (3).

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Abbreviations: DIG, digoxigenin; GH, glycoside hydrolase; nt, nucleotide(s); p*I*, isoelectric point; RACE, rapid amplification of cDNA ends.

Filamentous fungi are the most common industrial sources of xylanases because of their high productivity. In particular, GH family-11 xylanases from *Aspergillus niger* and *Trichoderma reesei* have been extensively studied, and the three-dimensional structures were shown to have the shape of a right hand (4, 5). In this study, we purified and characterized an extracellular GH family-11 xylanase from a filamentous fungus *Penicillium citrinum* FERM P-15944, which is the first *P. citrinum* xylanase to be reported. We also cloned and sequenced genomic DNA and cDNAs encoding the enzyme to clarify the organization of the encoding gene and to define the phylogenetic position of the fungal enzyme among homologous xylanases.

MATERIALS AND METHODS

Fungal strain and culture conditions *P. citrinum* FERM P-15944 used in this study is a wild-type strain originally isolated from soil samples by Hayashi *et al*. (6). The fungal strain was inoculated into 50 ml of medium (initial pH 6.5) containing 1% (w/v) birch-wood xylan (Sigma Chemical, St. Louis, MO, USA), 1.0% (w/v) yeast extract (Difco Laboratories, Detroit, MI, USA), 0.1% (w/v) K_2HPO_4, 0.06% (w/v) $MgSO_4 \cdot 7H_2O$, 0.05% (w/v) KCl, 0.001% (w/v) $FeSO_4 \cdot 7H_2O$, and 0.2% (w/v) agar in 500-ml Erlenmeyer flasks. The precultures were grown on a rotary shaker

(150 rpm) at 30°C for 2 d. A 2-ml aliquot of the culture was used to inoculate 100 ml of main-culture medium (initial pH of 7.5) that contained 1% (w/v) birch-wood xylan, 2.0% (w/v) yeast extract, 0.2% (w/v) K_2HPO_4, and 0.1% (w/v) NaCl in 500-ml Erlenmeyer flasks. Liquid cultures were grown on a rotary shaker (150 rpm) at 30°C for 3 d.

Enzyme and protein assays The reaction mixture consisted of 0.3 ml of a 1.0% (w/v) suspension of birch-wood xylan in deionized water, 0.1 ml of an enzyme solution, and 0.1 ml of 0.3 M acetate buffer (pH 5.0). After incubation at 50°C for 10 min, reducing sugars were determined by the Somogyi–Nelson method (7). One unit (U) of xylanase activity was defined as the amount of enzyme that liberated 1 μmol of xylose equivalents from xylan per minute. The purified xylanase was assayed for hydrolytic activity toward carboxymethyl cellulose (Wako Pure Chemical Industries, Osaka), under the conditions described above. The β-xylosidase activity was assayed by detecting the absorbance at 410 nm of *p*-nitrophenol released from the synthetic substrate *p*-nitrophenyl-β-D-xylopyranoside (5 mM; Sigma). Protein concentrations were measured by the method of Lowry *et al*. (8) with BSA (Sigma) as the standard.

Enzyme purification All purification procedures were carried out at 4°C. Submerged cultures were centrifuged at 2000×*g* for 30 min. The culture supernatant (2620 ml) was concentrated to 570 ml in dialysis tubing surrounded by a thick layer of dry polyethylene glycol (average molecular weight, 20,000). The concentrate was dialyzed for 2 d against several changes of distilled water, and lyophilized. The lyophilized sample was dissolved in 30 ml of 20 mM acetate buffer (pH 6.0) and loaded onto a DEAE-Cellulofine A-500 (Seikagaku Kogyo, Tokyo) column (2.6×45 cm) previously equilibrated with 20 mM acetate buffer (pH 6.0). The column was washed with the same buffer. The adsorbed proteins were eluted at a flow rate of 1.0 ml/min with a linear gradient of 0 to 0.6 M NaCl in the same buffer. The fractions exhibiting the enzyme activity were pooled and then chromatographed on a Superdex 75 pg (Amersham Biosciences, Piscataway, NJ, USA) column (1.6×60 cm) at a flow rate of 0.5 ml/min with 10 mM acetate buffer (pH 6.0) containing 0.15 M NaCl.

SDS–PAGE, amino acid sequencing, and isoelectric focusing The purified xylanase was subjected to SDS–PAGE using the method of Laemmli (9). Gels were stained for protein with Coomassie brilliant blue R-250. For the internal sequencing, the enzyme was digested with *Staphylococcus aureus* V8 protease (Wako). The N-terminal amino acid sequences of the intact protein and one of the resulting peptide fragments were identified using a G1005A Hewlett-Packard protein sequencer (Palo Alto, CA, USA) (10). The isoelectric point (p*I*) was measured by analytical isoelectric focusing with a Multiphor II electrophoresis system (Amersham Biosciences) as described previously (11).

Effect of pH and temperature on enzyme activity and stability The optimal pH for xylanase activity was determined by performing assays at 50°C for 10 min with the following buffers: 0.3 M acetate–HCl (pH 1.0 to 3.0), 0.3 M acetate (pH 3.0 to 6.0), 0.3 M phosphate (pH 6.0 to 8.0), and 0.3 M Tris–HCl (pH 8.0 to 10.0). The xylanase activity was assayed in a reaction mixture containing 0.1 ml of buffer of the desired pH, 0.3 ml of 1% (w/v) xylan suspension, and 0.1 ml of enzyme solution. Enzyme stability at different pH values was measured by the residual activity after the enzyme was incubated at room temperature for 3 h at pH 1.0 to 10.0. The optimal temperature for xylanase activity was found under the standard assay conditions except that the reaction mixture was incubated at temperatures from 30°C to 70°C. Thermal stability was measured in terms of the residual activity after the enzyme was incubated in 10 mM acetate buffer (pH 6.0) at temperatures from 30°C to 70°C for 30 min.

Effect of metal ions on enzyme activity The effect of metal cations on xylanase activity was determined at a final concentration of 1 mM. The reaction mixture containing 0.3 ml of 1% (w/v) xylan suspension, 0.1 ml of the enzyme solution, and 0.1 ml of 0.1 M PIPES buffer (pH 6.1) was incubated at 50°C for 10 min. The xylanase activity without any added compound was assigned a value of 100.

TLC analysis The reaction mixture (25 μl) consisting of equal volumes of a 1% (w/v) solution of xylooligosaccharide (xylobiose or xylotriose; Wako) or 10% (w/v) suspension of birch-wood xylan and the enzyme solution (6 U/ml) in 0.3 M acetate buffer (pH 5.0) was incubated at 50°C. Hydrolysis was stopped by boiling for 20 min, and the hydrolysis products were analyzed by TLC on silica gel plates (Merck, Darmstadt, Germany). The TLC plates were developed twice at room temperature with a solvent system of 1-butanol, pyridine, and water (3:2:1, v/v). Sugars were detected by spraying the plates with orcinol-sulfuric acid reagent and then heating at 120°C for 5 min.

DNA manipulations and analysis Restriction endonucleases and DNA-modifying enzymes were used as recommended by the supplier (Nippon Gene, Tokyo). Standard molecular cloning techniques were performed as described by Sambrook and Russell (12). PCRs were done in a thermal cycler (Takara Bio, Otsu). Plasmid pCR2.1-TOPO was used for TA cloning of amplified DNA fragments in *E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA). DNA sequencing of both strands was done with an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA) by using the primer-walking technique. The nucleotide and amino acid sequences were analyzed with the GENETYX-WIN software package (Software Development, Tokyo). Amino acid sequences were aligned with the ClustalW program. A neighbor-joining tree was constructed from the aligned amino acid sequences using the TREECON software package as described previously (13).

Construction of a xylanase-specific DNA probe A pair of degenerate 18-mer oligonucleotides was designed and synthesized according to the N-terminal and internal amino acid sequences of the purified enzyme (see Results): primer 1 (forward; 5′-YACYGG YACYWSYAAYGG-3′ derived from the STGTSNG in the N-terminus) and primer 2 (reverse; 5′-RTARGTRCCRAARTCYTC-3′ complementary to the nucleotide [nt] sequence encoding the EDF GTY in a V8 peptide). The PCR was done with the primer pair using genomic DNA from *P. citrinum* FERM P-15944 as a template under the same conditions as described before (10). The PCR-amplified fragment was separated by gel electrophoresis and purified from the gel. The purified DNA fragment was cloned into pCR2.1-TOPO and verified as a 388-bp internal fragment of the coding region of the potential xylanase gene, designated *xynA*, by sequencing. The purified PCR product was consequently labeled with digoxigenin (DIG) by the random-primed DNA-labeling and detection kit (Roche Diagnostics, Mannheim, Germany) for use as a *xynA*-specific hybridization probe.

Isolation of poly(A)$^+$ RNA and cDNA cloning Mycelia were harvested from 72-h grown cultures by filtration, and total RNA was isolated using an ISOGEN RNA isolation kit (Wako). Poly(A)$^+$ RNA was obtained from total RNA using a PolyATtract mRNA isolation system (Promega, Madison, WI, USA).

To map the 5′ and 3′ ends of the *xynA* transcripts and to obtain the full-length cDNA sequences, we did 5′ and 3′ rapid amplification of cDNA ends (RACE) using a SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA). First-strand cDNA was synthesized as described previously (11). PCR was done with each pair of gene-specific and universal primers using the first-strand cDNA as the template. For 5′ RACE, primer 3 (forward; UPM provided in the kit) and primer 4 (reverse; 5′-GTGCCGAAG TCTTCGACGATGTAG-3′ complementary to nt 468 to 485 relative to the A of the ATG codon [see nt sequence in Fig. 3]) were used. For 3′ RACE, primer 5 (forward; 5′-CTTCCAGCTCGACT