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Purification and properties of a family-10 xylanase from *Aureobasidium pullulans* ATCC 20524 and characterization of the encoding gene

Received: 25 March 2005 / Revised: 2 May 2005 / Accepted: 30 May 2005 / Published online: 30 June 2005
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Abstract An extracellular endo-1,4- β -xylanase was purified from the culture supernatant of the ascomycete *Aureobasidium pullulans* ATCC 20524 grown on xylan. The purified enzyme was homogeneous as judged by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and isoelectric focusing, which showed an apparent M_r of 39 kDa and a pI of 8.9, respectively. Xylanase activity was optimal at pH 6.0 and 70°C. The genomic DNA and cDNAs encoding this protein were cloned and sequenced. The xylanase gene (*xynII*) encoded a 26 amino acid signal peptide and a 335 amino acid mature protein. DNA regions encoding the signal sequence and the mature protein were interrupted by introns of 56 and 73 bp, respectively. The *xynII* 5'-noncoding region had two consensus binding sites (5'-GCCARG-3') for the transcription factor PacC mediating pH regulation. Quantitative real-time polymerase chain reaction analysis revealed that the transcription levels at pH 6.0 and 8.0 were 8-fold and 22-fold higher than that at pH 2.7, respectively. A cloned *xynII* cDNA was expressed and secreted in the yeast *Pichia pastoris*. Sequence alignment and phylogenetic analysis suggested that the XynII belongs to glycosyl hydrolase family 10 and that it is evolutionarily distant from two clusters formed by other family-10 xylanases.

Introduction

Xylan is a major constituent of hemicelluloses in the cell walls of monocots and hard woods (Puls and Schuseil 1993). It consists of a linear backbone of β -1,4-linked D-xylopyranose residues, which commonly contains side branches of arabinofuranose, glucuronic acid, and methylglucuronic acid. Efficient enzymatic hydrolysis of xylan is

essential for the industrially viable process that utilizes cellulosic residues. The attention on the potential applications of xylan-degrading enzymes has led to a finding of numerous microbial enzymes that differ in biochemical properties. Two key reactions proceed during hydrolysis of xylan; endo-1,4- β -xylanases (xylanases; 1,4- β -D-xylan xylanohydrolase, EC 3.2.1.8) cleave internal linkages in the xylan backbone to yield oligosaccharides, which are further hydrolyzed by β -xylosidases (1,4- β -D-xylan xylohydrolase, EC 3.2.1.37) to xylose. Many fungi, bacteria, and actinomycetes secrete multiple isozymes of xylanase as distinct gene products. These xylanases have been classified into two glycosyl hydrolase families 10 (formerly F) and 11 (formerly G) on the basis of amino acid sequence similarities (Henrissat 1991; Henrissat and Bairoch 1993). Family 11 consists of xylanases with a relatively low M_r ranging from 19 to 25 kDa, whereas the family 10 comprises xylanases with a higher M_r of >30 kDa. A hierarchy among the family-11 and -10 xylanases was proposed for microbial degradation of plant cell wall xylan to xylose (Pell et al. 2004). Therefore, a molecular characterization of such multiple xylanases produced by a single microorganism is important for a better understanding of their physiological roles in xylan degradation process.

The ascomycete *Aureobasidium pullulans* (de Bary) Arnaud, a saprophyte that occasionally occurs on leaf surfaces of plants, is a promising candidate for the application of genetic engineering (Deshpande et al. 1992). *A. pullulans* has been reported to produce a family-11 xylanase exhibiting high specific activity (Leathers 1986; Leathers 1989; Li et al. 1993; Ohta et al. 2001). The *A. pullulans* xylanase gene was cloned and sequenced (Li and Ljungdahl 1994; Ohta et al. 2001), and its cDNA was expressed in the yeast *Saccharomyces cerevisiae* or the methylotrophic yeast *Pichia pastoris* (Li and Ljungdahl 1996; Tanaka et al. 2004). To date, however, there have been no reports of family-10 xylanases from *A. pullulans*.

In the present paper, we describe the purification and properties of an extracellular family-10 xylanase from the fungal strain and the cloning and sequencing of a genomic DNA and cDNAs encoding the enzyme. Moreover, we

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defined the phylogenetic position of the *A. pullulans* xylanase among homologous xylanases belonging to family 10.

Materials and methods

Fungal strain and culture conditions

A wild-type strain *A. pullulans* var. *melanigenum* ATCC 20524 (Ohta et al. 2001) was used in this study. Growth medium contained 0.67% (w/v) yeast nitrogen base (Difco Laboratories, Detroit, MI, USA) and 1.0% (w/v) oat-spelt xylan (Sigma Chemical Co., St. Louis, MO, USA) as the sole carbon source in 0.1 M phosphate buffer (pH 6.0), unless otherwise stated. Liquid cultures were grown on a rotary shaker (150 rpm) in 500-ml Erlenmeyer flasks containing 100 ml of medium at 30°C for the time specified in the text.

Enzyme and protein assays

The reaction mixture consisted of 0.2 ml of a 1.0% (w/v) suspension of oat-spelt xylan in deionized water and 0.2 ml of a suitably diluted enzyme solution in 0.1 M sodium-acetate-HCl buffer (pH 6.0). After incubation at 45°C for 30 min, reducing sugars were determined by the method of Somogyi (1952) and Nelson (1955). One unit (U) of xylanase activity was defined as the amount of enzyme that liberated 1 μ mol of xylose equivalents from xylan per minute. To study the substrate specificity, the reaction mixture consisting of 0.5 ml of the enzyme solution and 0.5 ml of 10 mM *p*-nitrophenyl (*p*NP) glycosides (*p*NP- β -D-cellobioside, *p*NP- β -D-xylopyranoside, and *p*NP- α -L-arabinofuranoside; Sigma) was incubated at 45°C for 10 min. The reaction was stopped by the addition 1.0 ml of 1 M Na₂CO₃, and absorbance at 410 nm was measured. One unit (U) of enzyme activities toward *p*NP glycosides was defined as the amount of enzyme that liberated 1 μ mol of *p*-nitrophenol per minute from each substrate. Protein concentrations were measured by the method of Lowry et al. (1951), using bovine serum albumin (Sigma) as the standard.

Enzyme purification

The submerged cultures grown for 5 days were centrifuged for 30 min at 6,000 \times g at 4°C. The culture supernatant (1,900 ml) was concentrated to one tenth of its original volume in dialysis tubing surrounded by a thick layer of dry polyethylene glycol 20,000. The sample was further concentrated in an ultrafiltration cell by passage through a 3 \times 10³ molecular weight cutoff membrane (Diaflo YM3; Amicon, Beverly, MA, USA). The concentrate was subjected to cation exchange chromatography on a CM-Cellulofine C-500 (Seikagaku Kogyo, Tokyo, Japan) column (2.6 \times 45 cm) that had been equilibrated with 20 mM acetate

buffer (pH 6.0). The adsorbed proteins were eluted at a flow rate of 1.0 ml/min with a linear gradient of 0 to 1.0 M NaCl in the same buffer. The fractions exhibiting the enzyme activity were pooled and further purified by gel permeation chromatography on a Superdex 75 pg (Amersham Biosciences, Piscataway, NJ, USA) column (1.6 \times 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, N-terminal amino acid sequencing, and IEF

The purified enzyme was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Gels were stained for protein with Coomassie brilliant blue R-250. For the internal sequencing, the purified enzyme was cleaved by protease from *Staphylococcus aureus* V8 (Wako Pure Chemical Industries, Osaka, Japan). The resulting peptide fragments were separated by SDS-PAGE. The protein bands were electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA) as described previously (Nakamura et al. 1997). The N-terminal amino acid sequences of the intact protein and the resulting peptide fragments were identified using a Procise 492 protein sequencing system (Applied Biosystems, Foster City, CA, USA).

Analytical isoelectric focusing (IEF) was performed with a Multiphor II electrophoresis system (Amersham Biosciences) using Ampholine PAGplate (pH 3.5 to 9.5) according to the manufacturer's instructions. An IEF calibration kit (Amersham Biosciences) was used to determine the *pI* value.

TLC analysis

The reaction mixture (50 μ l) consisting of equal volumes of a 1% (w/v) solution of xylooligosaccharides (Megazyme, Wicklow, Ireland) or 3% (w/v) suspension of birch-wood xylan (Sigma) and the enzyme solution (20 mU/ml) in 50 mM phosphate buffer (pH 6.0) was incubated at 45°C. Hydrolysis was stopped by boiling for 10 min, and the hydrolysis products were analyzed for thin-layer chromatography (TLC) on silica gel plates (Merck AG, 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). Spots were stained by spraying the plates with orcinol-sulfuric acid reagent and then heating at 120°C for 5 min.

DNA manipulations and analyses

Restriction endonucleases and DNA-modifying enzymes were used as recommended by the supplier (Nippon Gene, Tokyo, Japan). Standard molecular cloning techniques were performed as described by Sambrook and Russel

(2001). Polymerase chain reactions (PCRs) were done in a thermal cycler (Takara Bio, Otsu, Japan). The nucleotide sequences were determined with an ABI Prism 310 genetic analyzer using the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems). A neighbor-joining tree was constructed from the aligned amino acid sequences using the TREECON software package as described previously (Akimoto et al. 2000).

Construction of a xylanase-specific DNA probe

A pair of degenerate 20-mer oligonucleotides was designed and synthesized according to the N-terminal amino acid sequence GLAQAWT and the internal amino acid sequence MKWESTE of the purified enzyme (see Results): primer 1 (forward) (5'-GGY CTY GCY CAG GCY TGG AC-3') and primer 2 (reverse) (5'-TCR GTR SWY TCC CAY TTC AT-3'). The primers amplified a 152-bp internal coding region of the potential xylanase gene, designated as *xynII*, from the *A. pullulans* genomic DNA as the template. The amplified fragment was cloned into pCR2.1-TOPO with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and sequenced to confirm its identity. The DNA fragment was then labeled with digoxigenin (DIG)-11-dUTP by the random-primed method using DIG DNA labeling and detection kit (Roche Diagnostics, Mannheim, Germany) for use as hybridization probe.

Isolation of poly(A)⁺ RNA and cDNA cloning

Mycelia were harvested from 72-h-old culture by centrifugation, and total RNA was isolated using an ISOGEN RNA isolation kit (Wako). Poly(A)⁺ RNA was obtained from total RNA by PolyAtract mRNA Isolation System IV (Promega, Madison, WI, USA). The 5' and 3' ends of *xynII* transcripts were determined by 5' and 3' rapid amplification of cDNA ends (RACE) using a SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) as described previously (Ohta et al. 2001). For 5' RACE, primer 3 (NUP provided in the kit) and primer 4 [5'-TCG GTA GAC TCC CAC TTC AT-3' complementary to nucleotides (nt) 285 to 304 relative to the A of the ATG start codon (see Fig. 3)] were used. For 3' RACE, primer 5 (5'-AGA ATG CCA TGA AGT GGG A-3' corresponding to nt 277 to 295) and primer 3 (see above) were used. The 5' and 3' RACE products were cloned into pCR2.1-TOPO and sequenced.

Quantitative real-time PCR

Mycelia were harvested from 72-h-old culture grown with pH control at pH 6.0 or 8.0 by 0.1 M phosphate buffer or without pH control (initial pH of 6.0). Total RNA was extracted from each mycelium as described above. First-strand cDNA was synthesized using total RNA as template and oligo d(T) primers by reverse transcriptase (RT)

(ReverTra Ace, Toyobo, Osaka, Japan). The *xynII* transcripts were quantified by real-time PCR with an ABI Prism 7000 (Applied Biosystems) using the first-strand cDNA as the template and SYBR Premix Ex Taq (Takara) according to the manufacturer's instructions. Quantification was based on a 104-bp amplicon generated using the following gene-specific primers: primer 6 (forward) (5'-CCA CTG ACG CGA AGC TCA A-3') and primer 7 (reverse) (5'-GGA AAC ACC CCA GAC AGT GAT AC-3') (the coding region interrupted by intron). The expression of a housekeeping 18S rRNA gene of *A. pullulans* (Li et al. 1996) as an endogenous reference was measured in parallel PCR runs using primer 8 (forward) (5'-TTG TCT GCT TAA TTG CGA TAA CGA-3') and primer 9 (reverse) (5'-GCT TGA GCC GAT AGT CCC TCT AA-3'). The thermal cycle program consisted of an initial step of 95°C for 10 s and 50 cycles of 95°C for 5 s and 60°C for 31 s. After determining amplifications, the target specificity was examined by the melting point analysis of RT-PCR products using a dissociation protocol, in which the temperature was raised linearly from 60°C. Each dissociation curve of the amplicons was observed as a single peak. In addition, amplicons of the expected size were confirmed by agarose gel electrophoresis. Real-time PCR data were processed using the relative quantitative method based on simulation of PCR kinetics (Liu and Saint 2002). Amplification efficiencies were calculated for each sample as

$$E = \left(\frac{R_{n,A}}{R_{n,B}} \right)^{\frac{1}{C_{T,A} - C_{T,B}}} - 1$$

where R_n is reporter fluorescence at cycle n , $R_{n,A}$ and $R_{n,B}$ are R_n at arbitrary thresholds A and B in an individual curve, respectively, and $C_{T,A}$ and $C_{T,B}$ are the threshold cycles at these arbitrary thresholds. Normalization of *xynII* expression level to that of the *rRNA* gene was presented as

$$\frac{R_{0,xynII}}{R_{0,rRNA}} = \frac{(1 + E_{rRNA})^{C_{T,rRNA}}}{(1 + E_{xynII})^{C_{T,xynII}}},$$

where $R_{0,xynII}$ and $R_{0,rRNA}$ are the initial copy numbers and $C_{T,rRNA}$ and $C_{T,xynII}$ are threshold cycles at the arbitrary thresholds. In this experiment, the *xynII* transcript level observed in *A. pullulans* grown without pH control was treated as the basal expression level.

Construction of yeast expression plasmid and *P. pastoris* transformation

The *xynII* cDNA encoding the precursor protein that includes a secretory signal sequence was amplified from the first-strand cDNA as the template with a pair of primers 5'-**GGAATTC**CA**TGC**ACTTCTCCACAATCAC-3' (forward) and 5'-**GGAATTC**CCT GAA TAC AAC TTG ATA CAT T-3' (reverse) (letters in bold type indicate the *xynII* coding sequence) containing *EcoRI* sites (under-

lined). The PCR product was cloned into the integrative yeast expression vector pPIC3.5 (Invitrogen) at *EcoRI* site in the orientation of transcription from *AOXI* promoter, yielding pXYN207. The pXYN207 was linearized with *SacI*, and the yeast *P. pastoris* GS115 (*his4*; Invitrogen) was transformed with the DNA fragment by electroporation as described previously (Tanaka et al. 2004).

Culture conditions for *P. pastoris* transformants and purification of recombinant enzyme

P. pastoris transformants were grown in 500-ml Erlenmeyer flask on an orbital shaker (150 rpm) at 30°C for 72 h. Each flask contained 100 ml of the buffered methanol complex medium as described previously (Tanaka et al. 2004). Additional methanol was supplied every 24 h to give a final concentration of 0.5% (v/v) during the culture period. Clear supernatant obtained from the contents of duplicate flasks was dialyzed against deionized water and lyophilized. The lyophilized sample was dissolved in 5 ml of 20 mM acetate buffer (pH 6.0) and subjected to purification by SP-Sepharose (Amersham Biosciences) and Superdex 75 pg column chromatographies.

Nucleotide sequence accession number

The nucleotide sequence of the 3,293-bp region containing the *xynII* gene will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB201542.

Results

Purification and properties of the xylanase

Table 1 summarizes the procedure for the purification of family-10 xylanase from *A. pullulans*. Cation exchange chromatography of the adsorbed proteins on a CM-Cellulofine column resulted in one major peak of xylanase with activity measured at pH 6.0. Meanwhile, most of the family-11 enzyme (XynI), the purification and character-

Table 1 Purification of extracellular GH family-10 xylanase from *A. pullulans*

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	186	301	0.6	1.0	100
Ultrafiltration	175	145	1.2	2.0	94
CM-Cellulofine	98.3	11.0	8.9	14.5	53
Superdex 75 pg	50.3	1.7	29.5	47.7	27

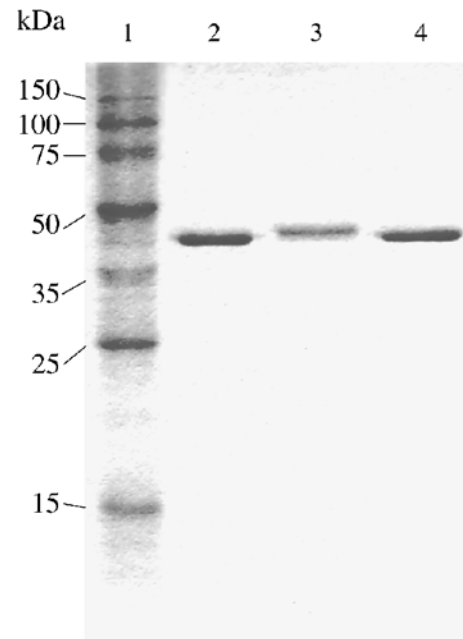


Fig. 1 SDS-PAGE of purified native xylanase from *A. pullulans* and the recombinant xylanases expressed in *P. pastoris*. Protein was visualized by Coomassie brilliant blue R-250 staining. Lanes (1), standard proteins; (2), the native xylanase; (3), the recombinant xylanase P-I; and (4), the recombinant xylanase P-II

ization of which have been previously reported (Ohta et al. 2001), was not adsorbed onto the column. The family-10 xylanase was eluted from a Superdex 75 pg column as a single protein peak that coincided with the peak of enzyme activity. This protocol afforded a 48-fold purification of the xylanase from the culture supernatant with a yield of 27%. The enzyme was homogeneous as judged by SDS-PAGE (Fig. 1) and IEF (data not shown), which showed an apparent M_r of 39 kDa and a pI of 8.9, respectively. The purified enzyme had a specific activity of 29.5 U/mg. The N-terminal amino acid sequencing identified the first 17 residues: S-Y-S-K-N-Q-G-L-A-Q-A-W-T-S-K-G-R. Internal sequences were identified for the two peptide fragments recovered after cleavage by V8 protease: N-A-M-K-W-E-S-T-E-P and L-D-V-R-F-T-T-P-A-T.

The xylanase activity was optimal at pH 6.0 and 70°C. The enzyme retained greater than 80% of the original activity between pH 4.0 and 10.0. The thermal stability was determined by incubating the enzyme solution in 0.1 M phosphate buffer (pH 6.0) at temperatures from 10 to 90°C for 30 min. The enzyme remained stable up to 70°C, but it lost the activity at 80°C. The xylanase activity was not influenced by ethylenediaminetetraacetic acid (112%), guanidine hydrochloride (110%), and *p*-chloromercuribenzoate (109%) at a final concentration of 1 mM, suggesting that any metal ions and sulfhydryl groups in the enzyme were not essential for the enzymatic reaction.

The purified xylanase was studied for its substrate specificity. The xylanase hydrolyzed birch-wood xylan (Sigma), with a similar activity toward oat-spelt xylan (Table 2). This enzyme also showed weak activities to-

Table 2 Substrate specificity of GH family-10 xylanase from *A. pullulans*

Substrate	Specific activity (U/mg)
Oat-spelt xylan	29.5
Birch-wood xylan	35.0
Carboxymethyl cellulose	ND
<i>p</i> NP- β -D-cellobioside	0.23
<i>p</i> NP- β -D-xylopyranoside	0.02
<i>p</i> NP- α -L-arabinofuranoside	<0.01

Experimental conditions are described in the text
 ND Not detected

ward *p*NP- β -D-cellobioside and *p*NP- β -D-xylopyranoside, but no detectable activity toward carboxymethyl cellulose and *p*NP- α -L-arabinofuranoside. TLC analysis indicated that initial hydrolysis of birch-wood xylan by the xylanase yielded a series of oligosaccharides with a degree of polymerization of two and above (Fig. 2a). Xylotriose was produced as an intermediate that was eventually cleaved to xylobiose and xylose. The enzyme hydrolyzed xylotriose, xylo-tetraose, and xylopentaose, but not xylobiose (Fig. 2b).

Cloning of the xylanase gene

The nucleotide sequence of the 152-bp fragment amplified from the *A. pullulans* genomic DNA (see **Materials and methods**) showed that the deduced amino acid sequence included the N-terminal and internal sequences of the purified xylanase. Southern blots of the genomic DNA digested with various restriction enzymes were probed with the DIG-labeled 152-bp fragment. A hybridized 9.0-kbp *Eco*RI DNA fragment containing the *xynII* gene was cloned into the *Eco*RI site of pUC18 to generate a plas-

Fig. 2 Thin-layer chromatogram of hydrolysis products from birch-wood xylan (a) and xylooligosaccharides (b) by purified xylanase from *A. pullulans*. The enzyme reaction was done as described in the text. Xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylo-tetraose (X_4), and xylopentaose (X_5) were used as standards (*St*)

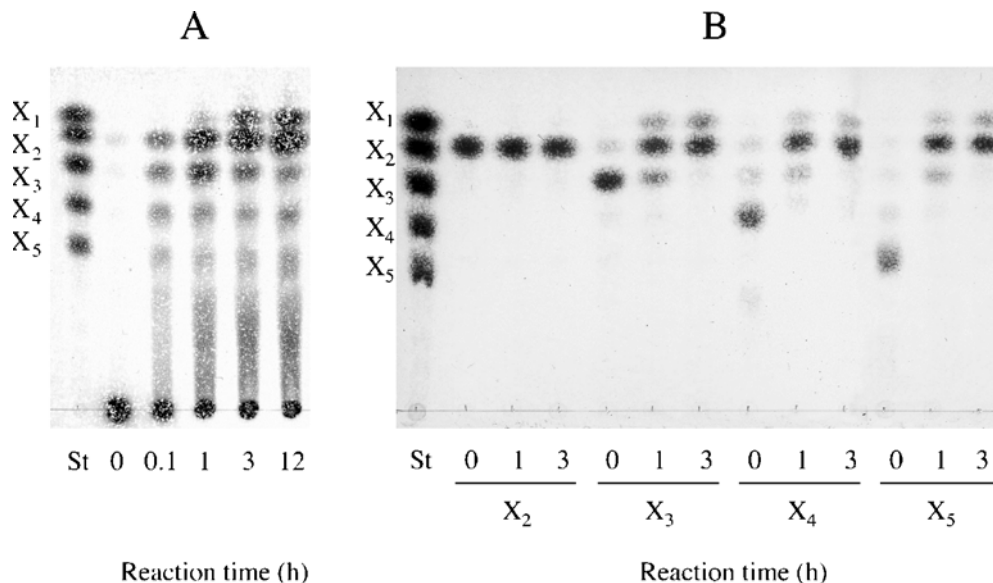


Fig. 3 Nucleotide sequence of *A. pullulans xynII* gene and its flanking regions and deduced amino acid sequence. The noncoding sequences, including introns, are shown by *lower case letters*. Two transcription start points and three polyadenylation sites are indicated by *open* and *solid arrowheads*, respectively. Two putative PacC binding sites in the *xynII* promoter region and a putative polyadenylation signal are *double* and *wavy underlined*, respectively. Amino acid sequences *underlined by thick line* were identical to those found for the purified protein. The putative catalytic Glu residues are indicated by *bold letters*. Potential sites for N-linked glycosylation are *underlined*. The positions of gene-specific primers, P1 to P7 (excluding the universal primer P3), are indicated by *horizontal arrows* above the sequence

mid pXYN204. The 3.8-kbp *Xba*I-*Kpn*I fragment from pXYN204 was subcloned into pUC18 to generate pXYN205.

Nucleotide sequences of the *xynII* gene and its cDNAs

The 3.8-kbp *Xba*I-*Kpn*I insert in pXYN205 was sequenced for the first 3,293-bp from the *Kpn*I site. The 3,293-bp sequence contained a complete *xynII* open reading frame and its flanking regions (Fig. 3). The first inflame ATG downstream of the transcription start points (see below) was the deduced start codon, which had a consensus A residue at the -3 position (Kozak 1989). A comparison of the *xynII* genomic and cDNA sequences showed that the regions encoding the signal sequence and the mature protein were interrupted by introns of 56 and 73 bp, respectively. Both introns fitted the GT/AG rule for 5' and 3' splicing sites (Gurr et al. 1987).

Two transcription start points were present at nt -60 (A) (three clones) and -54 (A) (three clones) as determined by sequence analysis of six independent cDNA clones of 5' RACE products. The *xynII* 5'-noncoding region did not contain the TATAAA and CCAAT motifs. A consensus binding site for the CreA repressor (5'-SYGGRG-3'), which mediates carbon catabolite repression in *Aspergil-*

ggtaccgctcctgcgactacacccaaaccggtgaagagcgagaaacacttctgttgcactgatcaagaccttcccgccatcttcacaaaccttttcga -1360
 cgagtcataoctccaccctccagacaccatccctctggactctccttatctctcccctggtatcgcaccaccgatctcctcgcgaagctctgcccag -1260
 gaaatcatcatgacacttgtaaatgggacatgctacttgccgagggtaaaagttccgcgaccgtctgatgggcaaaggcatcaacaagaatgtccact -1160
 acaaaatgattgaaggtgtgcctcatggctgggacaaggcacccaatcctctcaaaactactcctgggtcaaggagcattacctcaatgctaccaacca -1060
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 tacgtttctgcatagccgcttctaattgtctgcagtgccctctccggagagcagatgctgcgctctagcatgcaatttgattgcccgtggtgtcggataat -160
 ctgcccagacgaagtgcaaggtgaagtgtatgcggattgaatttatccagacaaaatacaaaagccccatccctctcattgatggggagatcttca -60

cagacagatttgcctcaatccagattccagcagtttttagtgcgtctgcaatcccacagatATGCACTTCTCCACAATCACCGCAGCTCTTGCCCTGCTTGG 41
 Δ M H F S T I T A A L A L L G 14

CCTGGTGTGCCACTCCAAGTAgtaggtcttttcttctcatcaattggcggccagaagtctgactttgaacctccagCTACAGCACCTCGTCTTACT 141
 L G A A T P T D Y S T S S Y S 29

CCAAGAATCAAGGACTTGCTCAGGCCTGGACTTCGAAAGGTCGTCAATACATTGGCACAGCACTGACTATTCGTGATGACCCCGTCGAACAAGGCATCAT 241
 K N Q G L A Q A W T S K G R Q Y I G T A L T I R D D P V E Q G I I 62

CCAATCGGAACCGATTTCAACTCCATAAACCAGAGAATGCCATGAAGTGGGAGTCTACCGAGCCGCAACGTAACAACCTTTACGTTGCTGGTGCCGAT 341
 Q S R T D F N S I T P E N A M K W E S T E P Q R N N F T F A G A D 95

GCCGTGCGCGACTTTGCCGACAGATAACAACAAGAGATGAGATGTACACTCTAGTTTGGCACTCCCAACTGCCTGCCTGGGTTAGTCAAGGCAACTTTG 441
 A V A D F A D R Y N K E M R C H T L V W H S Q L P A W V S Q G N F D 129

ACAACAAGACTTTGATCTCCATCATGGAGAACCACATCAAGAAAGTCGCTGGCAGATACAAGAACAAGTGTACCCACTGGGATGTTGTCAACGAAGCACT 541
 N K T L I S I M E N H I K K V A G R Y K N K C T H W D V V N E A L 162

CAATGAGGACGGCACTTACCGCAGCTCAGTCTTCTACAACACTATCGGAGAGGCATTCATCCCTATGCTTTCCGTTTCGCTGAGAAGTATGCGGGATCG 641
 N E D G T Y R S S V F Y N T I G E A F I P I A F R F A E K Y A G S 195

AAGACGAACTTTACTACAACGACTATAACCTTGAGTACGGCAGTGCCAAGGCCCTCGGTGCTCAACGCATCCTCAAGCTTGTTCAGAGCTATGGTGTCC 741
 K T K L Y Y N D Y N L E Y G S A K A L G A Q R I L K L V Q S Y G V Q 229

AAATCGATGGTGTGGGTTTGCAGGCTCACTTGAGTTCGAGGCTACTGCATCAACTGGCGCGGGTGTGACTCCTGACGTCCAAACCTTGACGAATGTGCT 841
 I D G V G L Q A H L S S E A T A S T G G G V T P D V Q T L T N V L 262

CAACTGTACACTGACCTTGGCGTCGAAGTTGCGTACACAGAGCTGGATGTGAGATTTACGACTCCCGCCACTGACGCGAAGCTCAAGGCTCAAGCGGAT 941
 K L Y T D L G V E V A Y T E L D V R F T T P A T D A K L K A Q A D 295

GCTTATGCCAGGGTGTTCAGTCTTGATCAACGTCAAGAGATGTGTTGGGATCACTGTCTGGGtaagttgcttctccttctcctgctgacgtgtttt 1041
 A Y A R V V Q S C I N V K R C V G I T V W 316

tcgttccaaaagtacactgctgatctttatatatagGGTGTTCGGACAAGTACTCTTGGATCCCTGGTGTTTTCCCGACTGAGGGTGCAGCTTTGCTCT 1141
 G V S D K Y S W I P G V F P T E G A A L L W 338

GGGATGAGAACTTCAATAAGAAGCCTGCTTACAGCTCTGTCTCAAGACCATTCAATCTTTCCGCAAACTTGAGcaaatcgttgagtgcggaaccgcaga 1241
 D E N F N K K P A Y S S V L K T I Q S F R K S * 361

acagacgaccatgatgaaataaaggaaagaatcgaggacgagtgcccgtagaggtagatgaatttctgaatgtatcaagttgtattcatgggctaaagct 1341
 ▲ ▲

gctgtcttgcgctcgctgtgagcacaggatcaagtctcaacaattgtgatagtcggcttgatcgaaggattattagttaacgaatgacttccgcgggtgc 1441
 ▲

tcatcttgggtgatagagaagcaacaattttagatccagcttggcagccttgaagaaggattcttggcagatgctagcaaatcttggcgtctgagatc 1541

cacatctgacattatcattctctacgattatctgactattcgcacatctctttagtgcttggaaacctgcatcttctctgacgtcaaatatcgagtcatt 1641

tgccgtgcttactatggcgtccaccaagcagcttgaagactcgcctacacgattcccaccacaatcgtgctgagcactcctaaagcggccagtg 1741

gccaatctactctctggttgaatacagacgaacagagtgcaaaagaactcgaagcggatcaaaaggactgaagacagtagagtaactctgt 1834

lus nidulans (Cubero and Scazzocchio 1994), was present at nt -294 (GTGGGG). In addition, consensus and near-consensus binding sites for the zinc finger transcription factor PacC (5'-GCCARG-3'), which mediates gene expression by the ambient pH in *A. nidulans* (Pañalva and Arst 2002), were present at nt -832 (GCCAAG) and -1136 (GCCATG of the complementary strand). However, the binding motif 5'-GGCTAAA-3' for the transcriptional activator XlnR of the xylanolytic system in *Aspergillus niger* (van Peij et al. 1998) was absent in the *xynII* promoter. Eight cDNA clones obtained by 3' RACE were polyadenylated at three different positions, 114 bp (two clones), 119 bp (four clones), and 138 bp (two clones) downstream of the stop codon. The consensus sequence AATAAA preceding the polyadenylation sites was present 44 bp downstream of the stop codon.

Modulation of the *xynII* mRNA levels by ambient pH

To investigate whether the *xynII* expression was regulated by the ambient pH, *A. pullulans* was grown for 72 h with pH control by 0.1 M phosphate buffer of pH 6.0 or 8.0 and without pH control. The initial pH 6.0 decreased to 2.7

without pH control, and the culture showed abundant growth and became black due to dark hyphae. The initial pH 6.0 or 8.0 remained constant under pH-controlled conditions, and the cultures showed delayed growth from that observed for the pH-uncontrolled culture and remained yellow or cream. Quantitative real-time PCR showed that the transcription levels at pH 6.0 and 8.0 were 8-fold and 22-fold higher, respectively, than that at pH 2.7 reached in the culture without pH control.

Deduced amino acid sequence and enzymatic activity of the *xynII* gene product

The *xynII* gene encoded a precursor protein (XynII) of 361 amino acids with a calculated M_r of 39,937 Da. The XynII contained sequences identical to the N-terminal and two internal amino acid sequences from the purified protein (see Fig. 3), indicating that the signal peptide of 26 amino acids was cleaved off during secretion. The XynII mature protein consisted of 335 amino acids with a calculated M_r of 37,344 Da and a deduced pI of 8.44. The calculated M_r was smaller than the 39 kDa estimated by SDS-PAGE, probably because the xylanase was a glycoprotein. In this

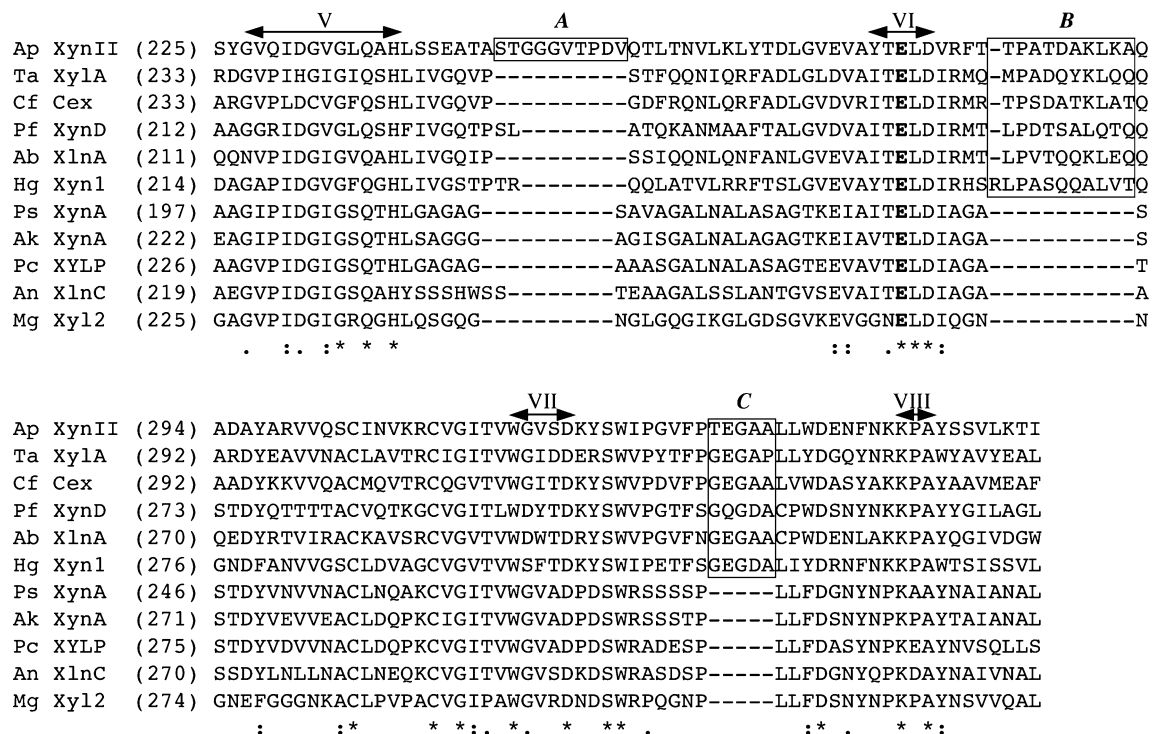


Fig. 4 Amino acid sequence alignment of the catalytic domain of *A. pullulans* XynII and other homologous xylanases. The alignment was done using the ClustalW program (version 1.81) and the aligned amino acid sequences depicted is the part encompassing the conserved regions V to VIII. *Ap* *A. pullulans* (this study), *Ta* *T. alba* (Z81013), *Cf* *C. fimi* (M15824), *Pf* *P. funiculosus* (AJ634957), *Ab* *A. bisporus* (Z83310), *Hg* *H. grisea* (AB001030), *Ps* *P. simplicissimum* (AF070417), *Ak* *A. kawachii* (D14847), *Pc* *P. chrysogenum* (M98458), *An* *A. nidulans* (Z49894), *Mg* *M. grisea* (L37530). Num-

bering of the amino acids shown in parentheses starts at the N-termini of the proteins. Gaps were introduced for optimal alignment and are indicated by dashes. Asterisks indicate identity, and single and double dots indicate semiconservative and conservative replacements. Conserved regions, V to VIII, are indicated by horizontal arrows. Inserted region A in *A. pullulans* xylanase and conserved regions B and C in xylanases of cluster I (see Fig. 5) are boxed. The putative catalytic Glu residues are indicated by bold letters

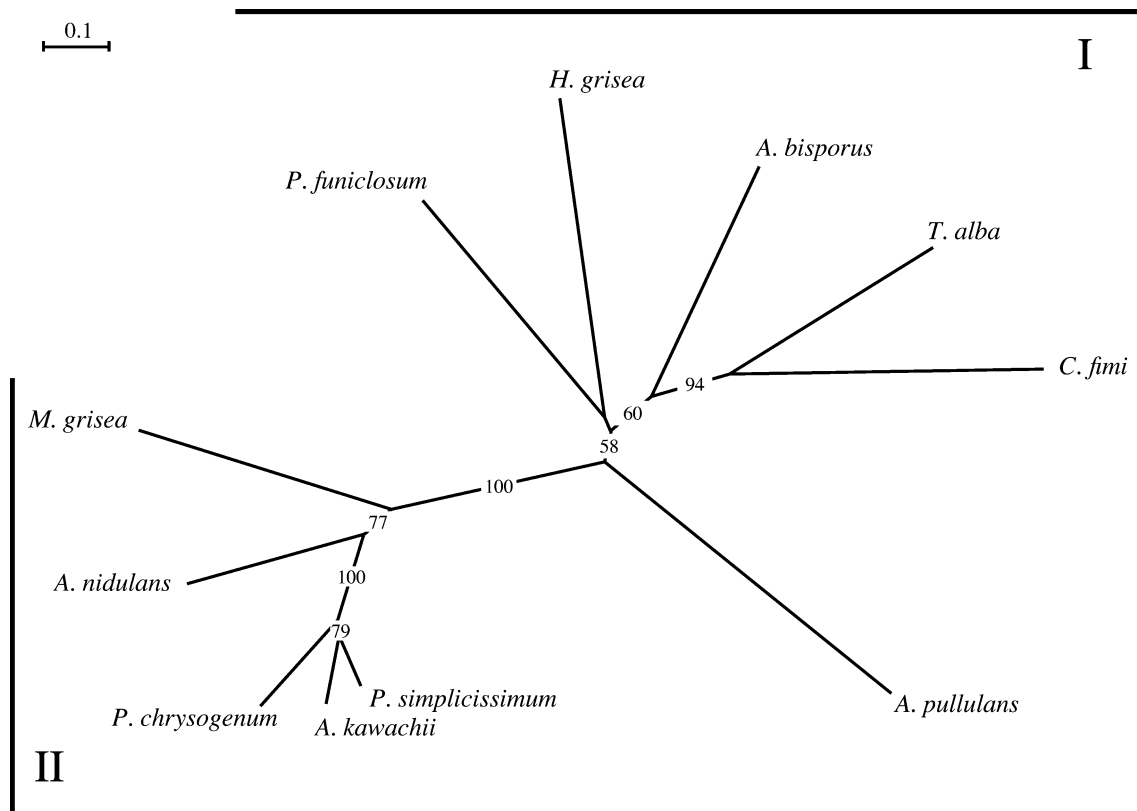


Fig. 5 Unrooted tree showing phylogenetic relationships of catalytic domains between *A. pullulans* XynII and other homologous xylanases. Numbers on the branch represent the percentage of bootstrap

confidence values based on 1,000 replications (values below 50 are not shown). The scale bar corresponds to 0.1 amino acid substitutions per site. For sources of sequence data, see Fig. 4

regard, there were two potential *N*-linked glycosylation sites (Asn-X-Thr) in the deduced amino acid sequence (see Fig. 3). The deduced *pI* was consistent with the *pI* of 8.9 found by IEF. Four Cys residues were present at positions 110, 152, 304, and 310.

To verify the identity of the *xynII* as a xylanase gene and to produce the recombinant enzyme in high yields, the yeast *P. pastoris* was chosen as a suitable host system for heterologous expression of *xynII* cDNA because of the lack of xylanase activity and its high secretion efficiency (Cregg 1999). The pXYN207 transformant grown for 72 h showed xylanase activity of 0.12 U/ml in the culture supernatant, whereas the control strain transformed with the vector pPIC3.5 was devoid of the extracellular enzyme activity. Elution profile of the extracellular proteins from an SP-Sepharose column revealed two xylanase peaks, P-I and P-II, and pooled fractions from each peak were purified individually by a Superdex 75 pg column. SDS-PAGE analysis showed that the M_r of the purified recombinant xylanase P-I was slightly larger than that of the P-II enzyme (Fig. 1). N-terminal amino acid sequence of P-II was identical to that of the native protein, whereas P-I contained additional six amino acid residues preceding the native cleavage site. Concentrations of the recombinant xylanases, P-I and -II, in the culture media were estimated to be 6.5 mg/l and 36 mg/l, respectively, on the basis of their specific activities. The results show that most of the XynII

precursor protein was correctly recognized and processed by the *P. pastoris* secretory pathway.

Discussion

We observed that when *A. pullulans* was grown on xylan as the only carbon source under the control at pH 6.0, the family-10 xylanase (XynII) is secreted into the culture media. The purified enzyme produced xylooligosaccharides as intermediates of xylan hydrolysis. It released mainly xylobiose and xylose as end products from xylan but not hydrolyzed xylobiose. These results indicate that the enzyme is an endo-1,4- β -xylanase. Family-10 xylanases exhibit lower substrate specificity than family-11 enzymes (Biely et al. 1997). The *A. pullulans* enzyme showed the weak activity toward *pNP*- β -D-xylopyranoside. The family-10 xylanase was free of activity toward carboxymethyl cellulose. This is advantageous for bleaching of pulps where purified cellulose is required.

The preferential transcription of the *xynII* gene at alkaline pH coincided with the presence of two PacC consensus sequences in the 5'-noncoding region. In contrast, the *xynI* gene encoding an acidophilic family-11 xylanase from the same *A. pullulans* strain (Ohta et al. 2001) was expressed at pH 2.7, but not or weakly expressed under the conditions of pH control at 6.0 or 8.0 (data not shown).

Likewise, the *A. nidulans xlnA* encoding a neutral xylanase and possessing two consensus PacC binding sites was preferentially expressed at alkaline ambient pH, whereas the *xlnB* gene encoding an acidic xylanase from the same strain was preferentially expressed at acidic ambient pH despite the presence of a single consensus PacC binding site (MacCabe et al. 1998). These results strongly suggested that the PacC-like transcription factor participates in pH regulation of the *xynII* expression in *A. pullulans*. Thus, *A. pullulans* seems to have a regulatory system responsible for the high transcription level at the optimum pH for the enzyme activity of the gene product.

A BLAST search of *A. pullulans* XynII in the protein sequence database found significant degree of identity to the following family-10 xylanases of fungal and bacterial origin that can hydrolyze xylan and/or cellulose (Gilkes et al. 1991): 46% for *Penicillium funiculosum* (accession no. AJ635947) and *Humicola grisea* (Iikura et al. 1997); 43% for the basidiomycete fungus *Agaricus bisporus* (De Groot et al. 1998) and the bacterium *Cellulomonas fimi* (O'Neill et al. 1986); 42% for the actinomycete *Thermobifida alba* (Blanco et al. 1997); 41% for *A. nidulans* (MacCabe et al. 1996); 39% for *Aspergillus kawachii* (Ito et al. 1992) and *Penicillium simplicissimum* (Schmidt et al. 1998); 38% for *Penicillium chrysogenum* (Haas et al. 1993); and 37% for the fungal pathogen *Magnaporthe grisea* (Wu et al. 1995). The alignment of catalytic domain of the homologous sequences showed eight conserved regions (I to VIII) as previously described for family-10 xylanases (Baba et al. 1994; Fukumura et al. 1995), suggesting that the XynII would be the first xylanase from *A. pullulans* belonging to family 10. The Glu-160 in region III (WDVVNE) and Glu-276 in region VI (TELD) of *A. pullulans* XynII could be involved in catalytic reaction as an acid-base and as a nucleophile, respectively (Tull et al. 1991). Cys-304 and -310 among the four Cys residues were highly conserved in homologous xylanases. Cys-110 and -152 might form the disulfide bond as viewed by a comparative protein modeling using Swiss-Model (Guex and Peitsch 1997; Peitsch 1995; Schwede et al. 2003) (data not shown).

Interestingly, both the *A. pullulans xynI* and *xynII* genes encoding family-11 and -10 xylanases, respectively, had a single intron in the region encoding the secretory signal sequence, whereas this is not the case for other homologous fungal xylanases. In addition, the *xynII* gene had only one intron in mature protein coding region despite the fact that typically several introns exist in fungal family-10 xylanase genes. Introns in family-10 xylanase genes have been shown to play a key role in protein evolution as a mediator for module fusion and exon shuffling to facilitate slightly different substrate specificities to xylanases without changing the catalytic function (Sato et al. 1999).

A phylogenetic tree was constructed for the catalytic domain using the alignment, the part of which is shown in Fig. 4. As shown by Sato et al. (1999), the above-mentioned xylanases except the *A. pullulans* XynII were separated into two clusters, I and II (Fig. 5). Cluster I included

bacterial and fungal xylanases in spite of their evolutionary distances and may have evolved through lateral or horizontal gene transfer of the catalytic domains, whereas cluster II consisted of those from only filamentous fungi. The XynII was phylogenetically located at an intermediate position between the two clusters.

Sequence alignment (see Fig. 4) showed that cluster I xylanases and the XynII shared extra regions B and C as compared to those of cluster II and a further insertion of ten amino acid sequences was found in the XynII (region A between conserved regions V and VI in Fig. 4). Although limited availability of closely related fungal xylanase sequences makes interpretation of the phylogenetic analysis difficult, the XynII seemed to be deviated from the cluster I xylanases and to be phylogenetically distant from cluster II xylanases.

In conclusion, this is the first report of the purification of a family-10 xylanase from *A. pullulans* as well as cloning and characterization of the encoding gene *xynII*. The *xynII* gene was preferentially expressed at alkaline ambient pH in contrast to the acid-expressed *xynI* gene encoding a family-11 xylanase from the same strain. Further compilation of the relevant sequence data to be published might allow us to present a more complete picture of the evolutionary relationship of the *A. pullulans* XynII with other family-10 xylanases.

Acknowledgements We thank Prof. Tatsuo Nakayama of Miyazaki Medical College for the N-terminal amino acid sequencing. This study was supported in part by The Iwatani Naoji Foundation's Research Grant 2003.

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