Purification and Properties of an Extracellular β-Xylosidase from *Aspergillus japonicus* and Sequence Analysis of the Encoding Gene

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Abbreviations: DIG, digoxigenin; GH, glycoside hydrolase; nt, nucleotide(s); ORF, open reading frame; *p*NP; *p*-nitrophenyl

A protein exhibiting β-xylosidase activity was purified from the culture filtrate of a filamentous fungus *Aspergillus japonicus* strain MU-2 grown on oat-spelt xylan. The purified enzyme was a glycoprotein with an apparent M_r of 113.2 kDa as determined by SDS–PAGE. β-Xylosidase activity was optimal at pH 4.0 and 70°C. The genomic DNA and cDNA encoding this protein was cloned and sequenced. Southern blot analysis indicated that the β-xylosidase gene (*xylA*) was present as a single copy in the genome. An open reading frame, consisting of 2412 bp, was not interrupted by introns, and it encoded a presumed signal peptide of 17 amino acids and a mature protein of 787 amino acids. The deduced amino acid sequence of the *xylA* gene product showed a high degree of identity to *Aspergillus niger* β-xylosidase XlnD (69%) that belongs to glycoside hydrolase family 3. The *xylA* gene fused in frame to the *Saccharomyces cerevisiae* α-mating factor signal peptide was functionally expressed in methylotrophic *yeast Pichia pastoris*. The recombinant *P. pastoris* strains showed β-xylosidase activity of 0.333 U/ml in the culture supernatant after 120-h cultivation.

[**Key words:** *Aspergillus japonicus*, β-xylosidase, extracellular enzyme, xylan]

INTRODUCTION

β-1,4-Xylan constitutes the major component of hemiellulose in the cell walls of monocots and hard woods and represents one of the most abundant biomass resources (1). Xylanolytic enzymes of microbial origin have received great attention in recent years due to their possible industrial applications, including bio-bleaching of kraft pulps and bio-mechanical pulping, bread making, fruit and vegetable processing, brewing, wine making, animal feed additives, and bio ethanol production (reviewed in Ref. 2).

β-1,4-Xylan is a heteroglycan with a backbone of β-(1→4)-linked D-xylopyranose residues that can be substituted to varying degrees with L-arabinofuranose, D-glucuronic acid, and/or 4-*O*-methyl-D-glucuronic acid as side chains at *O*-2 and *O*-3 positions. Two key reactions proceed during hydrolysis of the xylan backbone; endo-1,4-β-xylanases (xylanases; 1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8)

hydrolyze internal β-(1→4)-xylosidic linkages in the insoluble xylan backbone to yield soluble oligosaccharides, while β-xylosidases (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) cleave terminal xylose monomers from the non-reducing end of xylo-oligosaccharides. Additional enzymatic activities such as β-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid esterase, remove side-chain substituents.

Filamentous fungi are known to be efficient producers of xylanolytic enzymes, and several β-xylosidases of fungal origin have been purified and characterized (3-5). To date, a limited number of sequence information of β-xylosidase genes has been available from filamentous fungi, including *Aspergillus niger* (6), *Aspergillus awamori* (7), *Aspergillus oryzae* (8), *Talaromyces emersonii* (9), *Aspergillus nidulans* (10), *Trichoderma reesei* (11), and *Penicillium herquei* (12). β-Xylosidases are found in seven glycoside hydrolase (GH) families 3, 30, 39, 43, 51, 52, and 54, but fungal enzymes have been described so far only for families 3, 43 and 54.
(http://afmb.cnrs-mrs.fr/CAZY/).

In our previous study (13), *Aspergillus japonicus* strain MU-2 was used for extracellular production of β-fructofuranosidase. In this study, we purified an extracellular β-xylosidase from the *A. japonicus* and analyzed the primary structure. This is first report on extracellular GH family-3 β-xylosidase from *A. japonicus*.

MATERIALS AND METHODS

Fungal strain and culture conditions

A wild-type strain MU-2 used in this study was originally isolated from soil samples, and classified as *A. japonicus* (13). Liquid cultures (1200 ml) of the fungal strain were grown on a rotary shaker (150 rpm) in media (initial pH 7.0) containing 0.5% (w/v) oat-spelt xylan (Sigma Chemical Co., St. Louis, MO, USA), 0.5% (w/v) yeast extract, 0.2% (w/v) $NaNO_3$, 0.1% (w/v) K_2HPO_4, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.05% (w/v) KCl, and 0.001% (w/v) $FeSO_4 \cdot 7H_2O$ at 30°C for 7 d.

Enzyme assays, determination of kinetic parameters, and enzymatic deglycosylation

The reaction mixture consisting of 0.1 ml of the enzyme solution and 0.2 ml of 5 mM *p*-nitrophenyl (*p*NP)-β-D-xylopyranoside (Sigma) in 0.1 M acetate buffer (pH4.5) was incubated at 60°C for 10 min. The reaction was stopped by the addition 1.0 ml of 1 M Na_2CO_3, and absorbance at 410 nm was measured. One unit (U) of β-xylosidase activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per min from the synthetic substrate *p*NP-β-D-xylopyranoside. The activity of the purified β-xylosidase (0.02 U) toward various *p*NP-glycosides was determined as described above for β-xylosidase.

Xylanase activity was determined by the measurement of reducing sugars released from xylan by the Somogyi-Nelson method (12), as described previously (13). The purified β-xylosidase (0.02 U) was assayed for hydrolytic activity toward oligosaccharides and polysaccharides under the conditions described above.

Initial hydrolysis rates during the first 15-min reaction at 60°C were measured as a function of *p*NP-β-D-xylopyranoside concentrations ranging from 0.25 to 5.0 mM. Michaelis constants (K_m) and maximum velocity (V_{max}) were determined by fitting the initial hydrolysis rates to the Lineweaver-Burk equation.

N-deglycosylation of the purified enzyme was

performed by incubating the reaction mixture of 3 μl of 20 mM phosphate buffer (pH 7.0), 5 μl (10 μg) of purified enzyme solution, and 2 μl (0.4 U/ml) of N-glycosidase F solution (Roche Diagnostics, Mannheim, Germany) at 37°C overnight.

Enzyme purification

All purification procedures were carried out at 4°C. Submerged cultures were filtrated with suction through a Whatman GF/A glass micro-fiber. Solid ammonium sulfate was added to the filtrate with stirring to give 100% saturation and the mixture was left overnight. The precipitate was collected by centrifugation and dissolved in 20 mM McIlvain buffer (pH 5.0). The crude enzyme solution was subjected to anion-exchange chromatography on a DEAE-Toyopearl 650S (Tosoh Co., Tokyo) column (2.6 × 45 cm) that had been equilibrated with 20 mM McIlvain buffer (pH 5.0). The adsorbed proteins were eluted with a linear gradient of 0 to 1.0 M NaCl in the same buffer at a flow rate of 1.0 ml/min. The fractions exhibiting the enzyme activity were pooled and further purified by gel-permeation chromatography on a Superdex 200 pg (Amersham Biosciences, Piscataway, NJ, USA) column (1.6 × 60 cm) with 20 mM acetate buffer (pH5.0) containing 0.15 M NaCl at a flow rate of 0.5 ml/min.

Effect of pH and temperature on enzyme activity and stability

The optimal pH for β-xylosidase activity was determined using three different buffers: 0.1 M HCl–acetate (pH 2.0 to 5.0), 0.1 M acetate (pH 5.0 to 6.0), and 0.1 M phosphate (pH 6.0 to 9.0), 0.1 M glycine–NaOH (pH 9.0 to 11.0). Enzyme stability at different pH values was measured by the residual activity after the enzyme was incubated at 25°C for 3 h. The optimal temperature for β-xylosidase activity was found under the standard assay conditions except that the reaction mixture was incubated at temperatures from 30 to 80°C. The thermal stability was determined by incubating the enzyme solution in 0.1 M acetate buffer (pH 4.5) at temperatures from 20 to 80°C for 30 min.

SDS–PAGE and amino acid sequencing

The purified enzyme was subjected to SDS–PAGE using the method of Laemmli (13). Gels were stained for protein with Coomassie brilliant blue R-250. The enzyme was digested with *Staphylococcus aureus* V8 protease (Wako Pure Chemical Industries, Osaka) for the internal sequencing. The resulting peptide fragments were separated by SDS–PAGE. The protein bands were electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratory Hercules, CA, USA) as described previously (14). The N-terminal amino acid sequences of the intact protein and two peptide fragments were identified using a 476A gas-phase protein sequencer (Applied Biosystems, La Jolla, CA, USA).

Protein and carbohydrate determinations

Protein concentrations were measured by the method of Lowry *et al.* (15), using BSA (Sigma) as a standard. Total neutral carbohydrate was estimated by the phenol-sulfuric acid method of Dubois *et al.* (16), using glucose as a standard.

TLC analysis

The reaction mixture (100 μl) consisting of equal volumes of a 1.0% (w/v) solution of xylooligosaccharides (Megazyme, Wicklow, Ireland) and the enzyme solution (0.1 U/ml) in 50 mM acetate buffer (pH 4.5) was incubated at 50°C. Hydrolysis was stopped by boiling for 10 min, and the hydrolysis products were analyzed for 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, ethanol, and water (5:2:1, v/v). Spots were stained by spraying the plates with sulfuric acid-methanol reagent and then heating at 120°C for 5 min.

DNA manipulations and analysis

Genomic DNA of the strain MU-2 was extracted and purified from 72-h grown mycelium using an ISOPLANT DNA isolation kit (Wako). Restriction endonucleases and DNA-modifying enzymes were used as recommended by the supplier (Nippon Gene, Tokyo). Southern blot analysis was performed as described previously (16). Standard molecular cloning techniques were performed as described by Sambrook and Russell (17). PCRs were done in a thermal cycler (Takara Bio, Otsu). Plasmid pCR2.1 (Invitrogen, Carlsbad, CA, USA) was used for TA cloning of amplified DNA fragments in *E. coli* JM109. DNA sequencing of both strands was done with an ABI Prism 310 genetic analyzer (Applied Biosystems) by using the primer-walking technique. The nucleotide and amino acid sequences were analyzed by the GENETYX-WIN software package (Software Development, Tokyo).

Construction of a β-xylosidase-specific DNA probe

A pair of degenerate oligonucleotides was designed and synthesized according to the internal amino acid sequences ANTGNT and LKNDGT of the purified enzyme (see Results): primer 1 (forward; 5′-GCSAAYACYGGNAAY AC-3′) and primer 2 (reverse; 5′-GTNCCRTCR TTYTTNA-3′). The PCR was done with the primer pair by using genomic DNA from *A. japonicus* strain MU-2 as a template under the same conditions as described before (14). The PCR-amplified fragment was separated by gel electrophoresis and purified from the gel. The purified DNA fragment was cloned into pCR2.1 and verified as a 1016-bp internal fragment of the coding region of the potential β-xylosidase gene, designated *xylA*, by sequencing. The purified PCR product was consequently labeled with digoxigenin (DIG) by the random-primed DNA-labeling and detection kit (Roche) for use as a *xylA*-specific hybridization probe for Southern-blot analysis and genomic library screening.

Construction and screening of an *A. japonicus* genomic library

Hybridization data (see Results below) suggested that a single copy of β-xylosidase gene was present on an *Eco*RI-digested fragment of 4.9 kbp. Consequently, *Eco*RI digests of genomic DNA were fractionated by electrophoresis on a 1.0% (w/v) agarose gel. The gel segment containing DNA fragments of approximate size 4.9 kbp was excised. The DNA fragments were eluted and ligated into the *Eco*RI site of pUC18. *E. coli* JM109 cells were transformed with the ligation mixture to construct the genomic library that was enriched for the potential β-xylosidase gene. Transformant colonies were transferred to Hybond-N^+ nylon membranes and screened for hybridization with the *xylA*-specific DIG-labeled DNA probe.

Amplification of *xylA* cDNA by RT-PCR

Mycelia were harvested from 48-h grown cultures by filtration and ground to a fine powder under liquid nitrogen. Total cellular RNA was isolated from the powdered mycelia with an ISOGEN RNA isolation kit (Wako) by the method of Chomczynski and Sacchi (18). First-strand cDNA was synthesized with reverse transcriptase and oligo(dT)-anchor primer

provided in the 5′/3′ RACE kit (Roche). The *xylA* coding region was amplified by PCR with primer 3 (forward; 5′-ATGGCTGTGGCGGC TCTTGC-3′) and primer 4 (reverse; 5′-CTACT CATCCCCCGCCACCC-3′) using the first-strand cDNA as the template. The amplified 2.4-kbp fragment was separated by gel electrophoresis and purified from the gel. The purified 2.4-kbp fragment was cloned into pCR2.1 and sequenced.

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

The mature protein region of *xylA* gene was amplified from the cloned genomic DNA carried in pXYL130 as the template with primer 5 (forward; ′-CGGAATTC**CAACATAACAGCA GCTACGT**-3′) and primer 6 (reverse; 5′-ATA AGAATGCGGCCGC**CCTACTCATC**-3′) (letters in bold type indicate the *xylA* coding sequence). The primers contained additional sequences at their 5′ ends to generate *Eco*RI and *Not*I sites (underlined) at the 5′ and 3′ ends of the amplified fragment, respectively. An amplified *xylA* DNA fragment was digested with *Eco*RI and *Not*I, and cloned into the *Eco*RI-*Not*I sites of *P. pastoris* expression vector pPIC9 (Invitrogen) for fusion in frame to the signal peptide of *Saccharomyces cerevisiae* α-mating factor in the vector, yielding pXYL142. The pXYL142 was linealized with *Stu*I. The yeast *P. pastoris* GS115 (*his4*; Invitrogen) was transformed with the resulting DNA fragment by electroporation. His^+ transformants were recovered from minimal dextrose medium plates, and one of the transformants was randomly selected.

Expression of *A. japonicus xylA* gene in *P. pastoris* and purification of recombinant enzyme

P. pastoris transformants were grown at 30°C for 5 d in the buffered methanol-complex medium (pH 6.0) as described previously (22). The cells were removed by centrifugation at 10,000 × *g* for 10 min. The supernatant fluid was assayed for the β-xylosidase activity as described above. Recombinant β-xylosidase XylA was purified from the culture supernatant of pXYL142 transformant using DEAE-Sepharose Fast Flow (Amersham Biosciences) and Superdex 200 pg.

Nucleotide sequence accession number

The nucleotide sequence of the 4530-bp region containing the *xylA* gene will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB379633.

RESULTS AND DUSCUSSION

Purification of *A. japonicus* β-xylosidase

Table 1 summarizes the procedure for the purification of extracellular β-xylosidase from *A. japonicus* strain MU-2. β-Xylosidase was eluted from a Superdex 200 pg column as a single protein peak that coincided with the peak of enzyme activity. The purified β-xylosidase had a specific activity of 112 U/mg. This protocol afforded 59.4-fold purification of the enzyme from the culture filtrate with a yield of 12.3%. The purified enzyme was homogeneous as judged by SDS–PAGE, which showed an apparent M_r of 113.2 kDa (Fig. 1). The M_r of 113.2 kDa is within the commonly observed range of 90 to 132 kDa for other *Aspergillus* β-xylosidases (3, 5-8). The M_r decreased to 82.0 kDa after N-glycanase treatment as determined by SDS–PAGE analysis, suggesting the carbohydrate content of 27.6% (w/w). This value is in rough agreement with the total neutral

TABLE 1. Purification of extracellular β-xylosidase from *A. japonicus* strain MU-2

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	935	497	1.88	100	1.00
Ammonium sulfate precipitation	712	78.0	9.13	76.2	4.86
DEAE-Toyopearl 650S	181	6.65	27.0	19.3	14.3
Superdex 200 pg	115	1.03	112	12.3	59.4

carbohydrate content of 32.4% (w/w) determined by the phenol-sulfuric acid method. These observations together with the M_r estimation by native PAGE (data not shown) showed that *A. japonicus* β-xylosidase is a monomeric glycolprotein like equivalent enzymes from other *Aspergillus* spp. (5, 6). Attempts to identify the N-terminal amino acid sequence of the intact enzyme by Edman degradation were unsuccesful, presumably due to a blockade in the N-terminal amino acid. Instead, N-terminal amino acid sequences were determined for the two peptide fragments recovered after cleavage of the enzyme by V8 protease: LIANTGNTSP and GIVLLKNDGT.

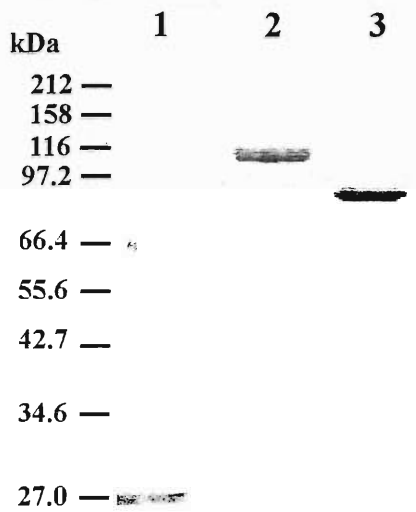

FIG. 1. SDS–PAGE of purified β-xylosidase from A. japonicus strain MU-2. Protein was visualized by Coomassie brilliant blue R-250 staining. Lanes: 1, standard proteins; 2, purified β-xylosdase ; 3, deglycosylated β-xylosidase by N-glycosidase

Enzymatic properties of β-xylosidase

β-Xylosidase activity of the purified enzyme was optimal at pH 4.0 and 70°C. The optimal pH was close to those previously reported for other *Aspergillus* β-xylosidases (5, 8). The enzyme retained greater than 90% of the original activity between pH 2.0 and 7.0 at room temperature for 3 h. The enzyme remained stable up to 60°C for 30 min, but it lost activity at 80°C (data not shown). The enzyme exhibited apparent K_m and V_{max} values of 0.314 mM and 114 μmol/mg/min, respectively, which are in the range of those previously reported for other β-xylosidase.

TLC analysis showed that the purified β-xylosdase hydrolyzed xylobiose and xylotriose to xylose after 24-h reaction (Fig. 2). The substrate specificity of the enzyme was determined using several *p*NP-glycosides, oligosaccharides, and polysaccharides (Table 2). In addition to the *p*NP-β-D-xylopyranoside, xylobiose and xylotriose that are typical substrates for β-xylosidases, the enzyme also had hydrolytic activities toward *p*NP-β-D-glucopyranoside (β-glucosidase activity) and *p*NP-α-L-arabinofuranoside (α-arabinofuranosidase activity). Similar broad specificity for glycosidic linkage type has been reported for other β-xylosidases from *A. niger* (3, 6) and

reesei (4). The enzyme weakly hydrolyzed xylans from oat spelt and birch wood, but it had no detectable activity against carboxymethyl-cellulose (CMC), crystalline cellulose (Avicel), and soluble starch. Like many other fungal β-xylosidases (7), the *A. japonicus* enzyme showed transglycosylation activity, especially at high substrate concentrations (data not shown). These results further confirmed that the *A. japonicus* enzyme possessed enzymatic properties characteristic for a β-xylosidase.

Nucleotide sequences of the *xylA* gene and its cDNAs

The 4.9-kbp *Eco*RI insert in pXYL130 was sequenced for the first 4530 bp from one of the *Eco*RI sites. The 4530-bp sequence contained a complete *xylA* open reading frame (ORF) of 2415 bp and its flanking regions (Fig. 3). The deduced start codon agreed with the consensus of a purine, preferably A, at nt position –3 and a G at nt position +4 in eukaryotic cells (23). Sequence comparison between cDNA and genomic DNA suggested that the *xylA* ORF was not interrupted by introns. This is also the case with other β-xylosidase genes from filamentous fungi (6, 8-10).

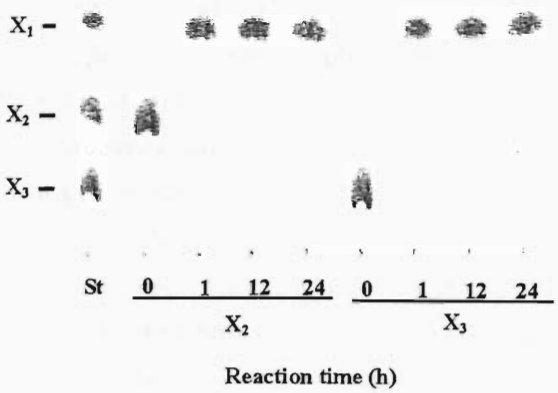

FIG. 2. TLC analysis for hydrolysis produts of xylobiose and xylotriose by purified β-xylosidase from *A. japonicus* strain MU-2.
The enzyme reaction was performed as escribed in the text. St, standards; X_1, xylose; X_2, xylobiose; X_3, xylotriose.

The *xylA* 5′-noncoding region included a putative TATA box (TATAAA) at nt –109 from the start codon. It has been shown that expression of xylanolytic enzymes in filamentous fungi is subject to induction and carbon catabolite repression. Carbon catabolite repression in *A. nidulans* is known to be

Table 2. Substrate specificity of extracellular β-xylosidase from *A. japonicus* strain MU-2

Substrate[a]	Concentration	Reaction time	Relative activity (%)
*p*NP-β-D-xylopyranoside	5 mM	10 min	100.0
*p*NP-β-D-glucopyranoside	5 mM	10 min	31.6
*p*NP-α-D-xylopyranoside	5 mM	10 min	0.0
*p*NP-α-D-glucopyranoside	5 mM	10 min	0.0
*p*NP-β-D-galactopyranoside	5 mM	10 min	0.0
*p*NP-α-D-galactopyranoside	5 mM	10 min	0.0
*p*NP-α-L-arabinofuranoside	5 mM	10 min	17.7
Oat-spelt xylan	1% (w/v)	6 h	0.7
Birch-wood xylan	1% (w/v)	6 h	2.0
Cellobiose	5 mM	6 h	1.5
Xylobiose	5 mM	6 h	3.7
Xylotriose	5 mM	6 h	6.0

[a] No activity was detected for CM-cellulose, Avicel, soluble starch, sucrose maltose, lactose, and cellobiose.

mediated via the transcriptional repressor protein CREA (24). Five consensus binding sites for the CreA repressor (5′-SYGGRG-3′) were present further upstream in the *xylA* promoter region at nt –153 (GCGGGG of the complementary strand), –164 (GCGGAG of the complementary strand), –276 (CCGGAG of the complementary strand), –455 (CTGGAG), and –687 (CTGGAG of the complementary strand). The sequence 5′-GGCTAA-3′ known as a potential binding site for a transcriptional activator XlnR of the xylanolytic system in *A. niger* (25) was present at nt –195 (TTAGCC of the complementary strand), –219 and –272 in the *A. japonicus xylA* gene. Direct repeats of a sequence 5′-TCCTCAACTCC-3′ were found in the *xylA* 5′-noncoding region at nt –310 to –300, –299 to –289, and –288 to –278. In common with some other fungal genes, the typical polyadenylation signal, AATAAA, was not present within the 5′-noncoding region.

Deduced amino acid sequence of the *xylA* gene product

The *xylA* gene encoded a precursor protein (XylA) of 804 amino acid residues (Fig. 3). Internal sequences determined for the two peptide fragments were found in residues 82 to 89 and 413 to 422 of the deduced amino acid sequence. A potential signal peptidase recognition site was predicted between Gly-17 and Gln-18 by SigmalP program (26). It is likely that the N-terminal Gln residue of the mature enzyme subsequently cyclized to pyroglutamate, yielding a blocked N-terminus. The XylA mature protein consisted of 787 amino acids with a calculated M_r of 84,638 Da and a deduced isoelectric point of 4.64. The calculated M_r was in good agreement with the M_r 86.0 kDa measured by SDS–PAGE for the deglycosylated enzyme. The XylA protein possessed 18 potential *N*-glycosylation sites (Asn-X-Ser/Thr). The M_r 113.2 kDa measured by SDS–PAGE for the purified enzyme implies that some of the potential *N*-glycosylation sites are glycosylated.

Sequence comparison with other β-xylosidases

A BLAST search of *A. japonicus* XylA in the protein sequence databases found significant degrees of identity to the following GH family-3 β-xylosidases: *A. niger* XlnD (69%) (6), *A. awamori* Xaw1 (69%) (7), *Aspergillus fumigatus* XylA (69%) (CM000169; locus tag AFUA_1G16920), *A. oryzae* XylA (68%) (8), *T. emersonii* Bxl1 (66%) (9), *A. nidulans* XlnD (65%) (10), *T. reesei* Bxl1 (64%) (11), *Aspergillus clavatus* β-xylosidase (63%) (DS027059; locus tag ACLA_018590), and *Neosartorya fischeri* (synonym: *Aspergillus fischeri*) β-xylosidase (48%) (DS027697; locus tag NFIA_003180). The *A. japonicus* XylA also showed significant identities of the amino acid sequence to the β-glucosidases belonging to GH family 3. The deduced amino acid sequence similarity together with its biochemical properties described above suggested that the *A. japonicus* XylA belongs to GH family 3. As deduced from the Arabidopsis β-xylosidase classified in GH family 3 (27), Asp-307 and Glu-516 in *A. japonicus* XylA were expected to be involved in the catalytic reaction as a nucleophile and an acid/base, respectively. The conserved WGR and KH motifs in GH family 3, beginning at residues Trp-180 and Lys-222 for *A. japonicus* XylA, are thought to be involved in substrate binding.

Expression and secretion of β-xylosidase in *P. pastoris*

To prove the identity of the *xylA* as a β-xylosi-

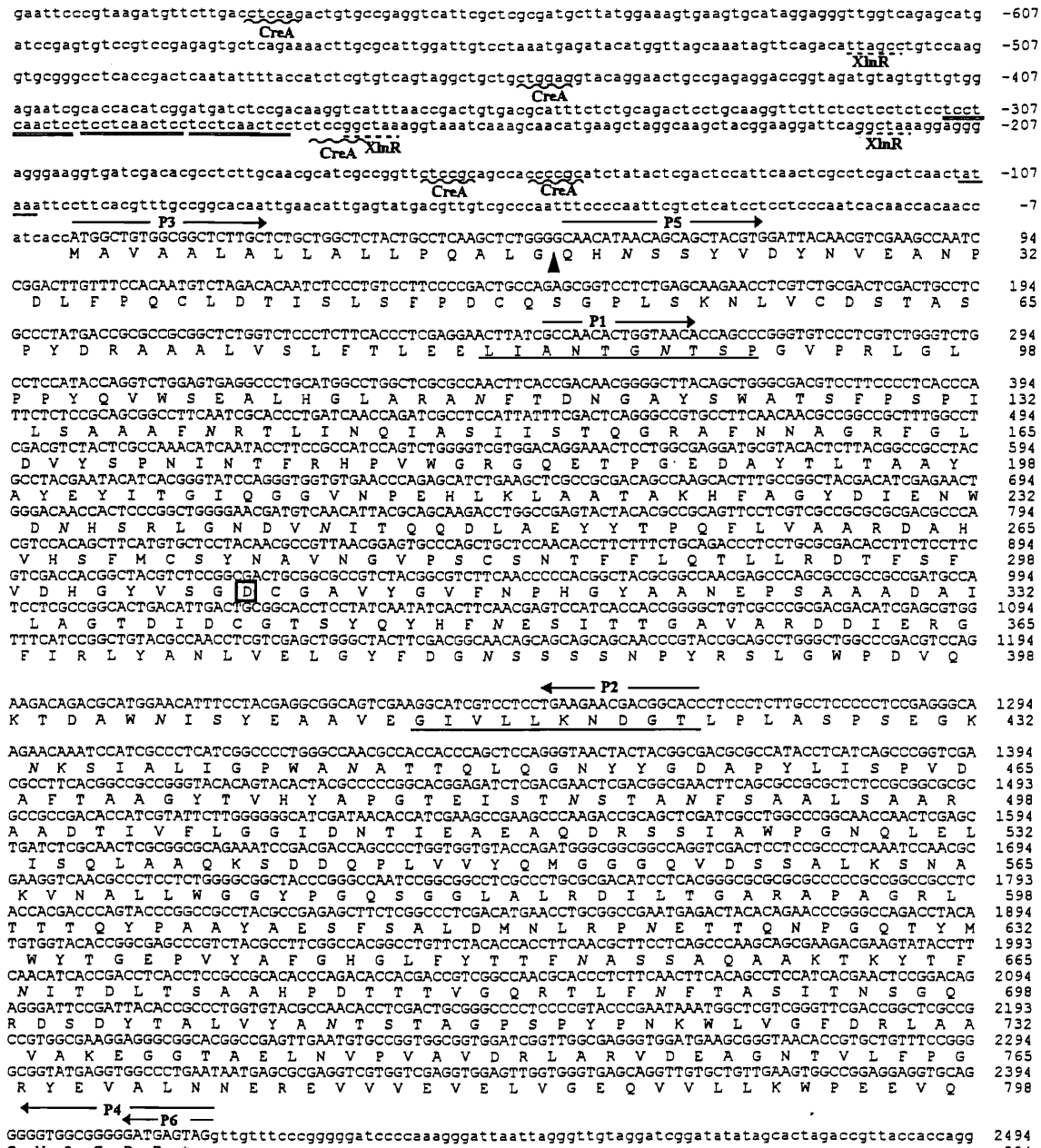

FIG. 3. Nucleotide and deduced amino acid sequences of *xylA* gene encoding β-xylosidase from *A. japonicus* strain MU-2. Numbers in the right-hand column refer to the nt sequence and are relative to the A of the start codon, and numbers on the right represent amino acid residues. Underlined amino acid sequences were identical to those found for the purified protein and its peptide. The non-coding sequences are shown in lower-case letters. Solid upward arrowheads indicate putative signal peptide cleaving sites. The positions of gene-specific primers, P1 to P6, are indicated by horizontal arrow. The predicted TATA box is underlined. Five putative CreA-binding sites (5′-SYGGRG-3′) and three putative XlnR-binding sites (5′-GGCTAA-3′) are indicated by wavy and dotted underlines, respectively. The direct repeats of a sequence were indicated by double underlines. A putative catalytic residue Asp-329 was boxed. Potential *N*-glycosylation sites are in italics. The stop codon is indicated by an asterisk.

dase gene and to produce the recombinant enzyme in high yields, we studied the expression of the *xylA* gene in *P. pastoris* GS115. The pXYL142-derived transformant carrying *xylA* gene and the control strain transformed with the vector plasmid pPIC9 showed similar growth curves in shake-flask cultures (Fig. 4A). The pXYL142 transformant grown for 120 h showed β-xylosidase activity of 0.333 U/ml in the culture supernatant, whereas the culture supernatant of the control transformant had the low level of activity with *p*NP-β-D-xylopyranoside that can be regarded as insignificant. SDS–PAGE revealed that the recombinant XylA showed an apparent M_r of 118.4 kDa, which was slightly larger than 113.2 kDa of the native enzyme from *A. japonicus* (Fig. 4B). The difference in M_r between the native and recombinant enzymes may be ascribed to the presence of additional oligosaccharide residues in the latter. TLC analysis showed that the purified recombinant XylA hydrolyzed xylo-oligosaccharide to xylose at the same level as native XylA from *A. japonicus* (data not shown). Thus, the results showed that the pXYL142 transformant expressed functional XylA.

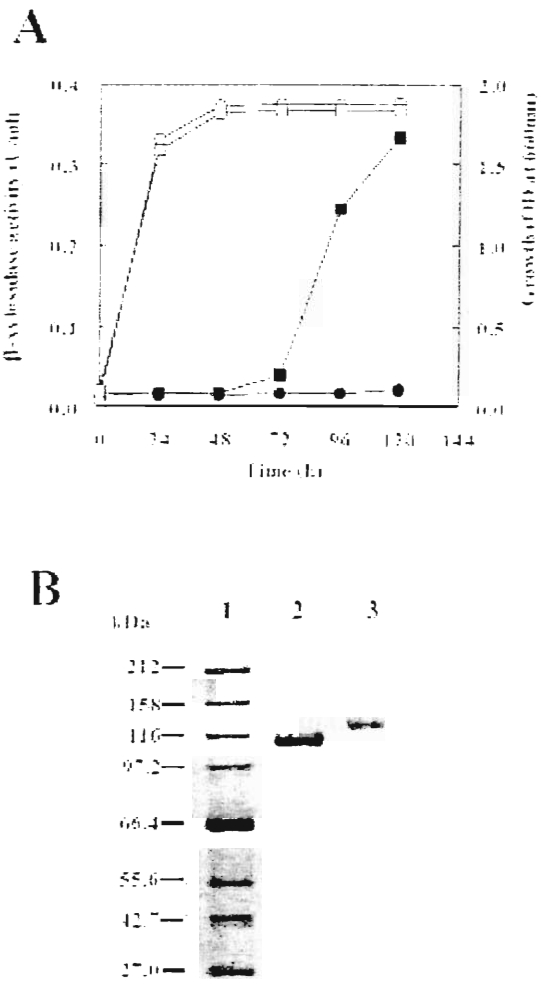

FIG. 4. (A) Growth (open symbols) and extracellular β-xylosidase activity (solid symbols) in shake-flask cultures of *P. pastoris* transformants. The pXYL142 transformant carrying mature protein region of *xylA* gene (symbols: square) and the control transformant lacking *xylA* gene (symbols: circles) were grown at 30°C in the buffered methanol complex medium (pH 6.0). (B) SDS–PAGE analysis to detect extracelluar recombinat xylanase. The recombinant β-xylosidase was purified from supernatant fluids of the pXYL142 transformant and loaded on the gel. Lanes: 1, standard proteins; 2, the recombinant β-xylosidase; 3, the native β-xylosidase.

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