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Cell-associated β-xylosidase from *Aureobasidium pullulans* ATCC 20524: Purification, properties, and characterization of the encoding gene

Kazuyoshi Ohta,* Hirohisa Fujimoto, Shinya Fujii, and Motoki Wakiyama

Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai Nishi, Miyazaki 889-2192, Japan

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A cell-associated enzyme exhibiting β-xylosidase activity was purified from the cell extract of the dimorphic fungus, *Aureobasidium pullulans* strain ATCC 20524, grown on oat spelt xylan. The purified enzyme was homogeneous as judged by SDS–PAGE, which showed an apparent M_r of 88.5 kDa. β-Xylosidase activity was optimal at pH 3.5 and 70 °C. The enzyme exhibited apparent K_m and V_{max} values of 3.5 mM and 263 μmol/mg/min, respectively, for *p*-nitrophenyl-β-D-xylopyranoside. The enzyme also showed some α-L-arabinofuranosidase activity. Southern blot analysis indicated that the β-xylosidase gene (*xylI*) was present as a single copy in the genome. The genomic DNA and cDNA encoding this protein were cloned and sequenced. The open reading frame of 2415 bp was interrupted by two introns of 54 and 52 bp, and it encoded a presumed signal peptide of 20 residues and a mature protein of 785 residues with a calculated M_r of 85,045 Da and a deduced isoelectric point of 4.57. The protein was *N*-glycosylated and possessed 16 potential *N*-glycosylation sites. Two distinct transcription start points of the *xylI* gene were present at nt −32 (A) and −26 (A) from the start codon. The *xylI* cDNA was functionally expressed in the yeast *Pichia pastoris*. The deduced amino acid sequence of the *xylI* gene product was 49% identical to the *Talaromyces emersonii* β-xylosidase Bxl1, which belongs to the glycoside hydrolase family 3.

[**Key words:** *Aureobasidium pullulans*; Cell-associated enzyme; Glycoside hydrolase family 3; Xylan; β-Xylosidase]

β-1,4-Xylan is a heteroglycan with a backbone of β-(1→4)-linked D-xylopyranose residues that can be substituted with L-arabinofuranose, D-glucuronic acid, and/or 4-*O*-methyl-D-glucuronic acid (1). It constitutes the major component of hemicelluloses found in the cell walls of monocots and hard woods and represents one of the most abundant biomass resources. Recently, xylanolytic enzymes of microbial origin have received great attention due to their possible industrial applications for sustainable fuel–ethanol production from xylan. Two key reactions proceed during hydrolysis of the xylan backbone; endo-1,4-β-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 xylooligosaccharides, while β-xylosidases (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) are exoglycosidases that cleave terminal xylose monomers from the non-reducing end of short-chain xylooligosaccharides (2). Additional enzyme activities such as α-L-arabinofuranosidase, α-D-glucuronidase, and acetyl xylan esterase, remove side-chain substituents.

The dimorphic fungus *Aureobasidium pullulans* has been recognized as an excellent producer of xylanolytic enzymes. We have recently purified glycoside hydrolase (GH) family-10 and -11 xylanases from the culture filtrate of *A. pullulans* strain ATCC 20524 and cloned the respective encoding genes (3, 4). An extracellular β-xylosidase was previously purified from the culture filtrate of *A. pullulans* strain CBS 58475 (5). However, β-D-xylosidase activity of *A. pullulans* is primarily localized in the periplasmic space (6). Here, we describe the purification and properties of a cell-associated β-xylosidase from the *A. pullulans* strain ATCC 20524, which appears to differ from the extracellular enzyme previously reported for the strain CBS 58475 (5) not only in its cellular location but also in its kinetic and molecular properties. To date, there have been no reports of β-xylosidase genes from *A. pullulans*. We also report the organization of the β-xylosidase gene (*xylI*), the primary structure of the enzyme, and its expression in the methylotrophic yeast *Pichia pastoris*.

MATERIALS AND METHODS

Fungal strain and culture conditions A wild-type strain *A. pullulans* var. *melanigenum* ATCC 20524 (3) was used in this study. Growth medium (initial pH of 5.0) contained 0.67% (w/v) yeast nitrogen base without amino acids (Sigma Chemical Co., St. Louis, MO, USA) and 1.0% (w/v) oat spelt xylan (Sigma) as the carbon source. Liquid cultures were grown in a 500-ml shake flask with 100 ml of medium on a rotary shaker (120 rpm) at 30 °C for 5 days, unless otherwise stated.

Enzyme and protein assays The reaction mixture consisting of 0.1 ml of appropriately diluted enzyme solution, 0.2 ml of 5 mM the synthetic substrate *p*-nitrophenyl (*p*NP)-β-D-xylopyranoside (Sigma) in deionized water, and 0.7 ml of 0.1 M acetate–HCl buffer (pH 3.5) was incubated at 70 °C for 10 min. The reaction was stopped by adding 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 *p*NP-β-D-xylopyranoside. Protein concentrations were measured by the method of Lowry et al. (7), using BSA (Sigma) as a standard.

Abbreviations: DIG, digoxigenin; GH, glycoside hydrolase; nt, nucleotide(s); ORF, open reading frame; *p*NP, *p*-nitrophenyl; RACE, rapid amplification of 5′ and 3′ cDNA ends.

* Corresponding author. Tel./fax: +81 985 58 7217.
E-mail address: k.ohta@cc.miyazaki-u.ac.jp (K. Ohta).

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Substrate specificity The hydrolytic activity of the purified β-xylosidase toward other *p*NP-glycosides was determined as described above for assay of β-xylosidase activity, except that the reaction mixture consisting of 0.1 ml of enzyme solution (0.006 U of β-xylosidase activity), 0.2 ml of 10 mM *p*NP-glycoside (Sigma) in deionized water, and 0.7 ml of 0.1 M acetate–HCl buffer (pH 3.5) was incubated for 30 min. To determine the capacity of the purified enzyme to hydrolyze various oligo- and polysaccharides, the reaction mixture consisting of 0.1 ml of enzyme solution (0.02 U of β-xylosidase activity), 0.25 ml of 4 mM oligosaccharides or 2% (w/v) polysaccharides in deionized water, and 0.65 ml of 0.1 M acetate–HCl buffer (pH 3.5) was incubated at 50 °C for 6 h. The increase in reducing sugars released was determined using the Somogyi–Nelson method (8).

Determination of kinetic parameters Initial hydrolysis rates during the first 10 min of the reaction at 70 °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.

Enzyme purification All purification procedures were conducted at 4 °C. Mycelia were harvested from the liquid culture by centrifugation at 9000×*g* for 5 min, washed with deionized water, and lyophilized. The lyophilized mycelia (17.7 g) were suspended in 500 ml of 50 mM maleate buffer (pH 5.5) containing 0.5% (w/v) lytic enzyme toward the cell wall of filamentous fungi (Yatalase; Takara Bio, Otsu, Japan) and 0.5 M $MgSO_4$. The reaction mixture in a 1-liter Erlenmeyer flask was incubated on a rotary shaker (120 rpm) at 30 °C for 5 h and sonically disrupted with twenty 30-s pulses with 60-s intervals of cooling on ice. The resulting lysate was centrifuged at 5000×*g* for 10 min. The supernatant solution (430 ml) was concentrated to 93 ml in a dialysis tube surrounded by a thick layer of dry polyethylene glycol 20,000. Subsequent chromatographic purification of enzyme was performed on a Bio-Rad BioLogic DuoFlow system (Hercules, CA, USA) as shown below.

Anion exchange chromatography The concentrate was loaded onto on a DEAE Sepharose Fast Flow (GE Healthcare, Buckinghamshire, UK) column (3.0×40 cm) that had been equilibrated with 20 mM phosphate buffer (pH 6.0). The adsorbed proteins were eluted with a linear gradient of 0–1.0 M NaCl in the same buffer at a flow rate of 1.0 ml/min.

Cation exchange chromatography The fractions exhibiting the enzyme activity were pooled and then purified on an SP Sepharose Fast Flow (GE Healthcare) column (3.0×50 cm) that had been equilibrated with 20 mM acetate–HCl buffer (pH 3.0). Elution was performed with a linear gradient of 0–1.0 M NaCl in the same buffer at a flow rate of 1.0 ml/min.

Gel permeation The fractions exhibiting the enzyme activity were pooled and further loaded onto a Superdex 200 pg (GE Healthcare) column (4.0×100 cm) that had been equilibrated with 20 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 0.5 ml/min. β-Xylosidase was finally eluted as a single peak of protein that coincided with that of enzyme activity.

The fractions exhibiting the enzyme activity were pooled and desalted by Sephadex G-25 Superfine (GE Healthcare) column (1.6×2.5 cm) with 20 mM phosphate buffer (pH 6.0) at a flow rate of 3.0 ml/min.

Effects of pH and temperature on enzyme activity and stability The pH and temperature profiles of the enzyme were determined as previously described (9), except the use of 20 mM phosphate buffer (pH 6.0) to determine the thermal stability instead of 0.1 M acetate buffer (pH 4.5).

Enzymatic *N*-deglycosylation The purified enzyme was treated with *N*-glycosidase F (Roche Diagnostics, Mannheim, Germany) according to the procedures described previously (9).

SDS–PAGE and amino acid sequencing The purified enzyme was subjected to SDS–PAGE using the method of Laemmli (10). Gels were stained for protein with Coomassie brilliant blue R-250. For internal sequencing, the enzyme was digested with *Staphylococcus aureus* V8 protease (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 (Millipore, Billerica, MA, USA) as previously described (11). The N-terminal amino acid sequences of the intact protein and one of the resulting peptide fragments were identified using a Procise 494 HT protein sequencing system (Applied Biosystems, Foster City, CA, USA).

DNA manipulations and analysis Fungal genomic DNA was extracted and purified from mycelia grown for 72 h using an ISOPLANT DNA isolation kit (Wako). Southern blot analysis was performed as previously described (11). Standard molecular cloning techniques were performed as described by Sambrook and Russell (12). PCRs were done in a thermal cycler (Takara Bio). Plasmid pCR2.1 (Invitrogen, Carlsbad, CA, USA) was used for TA cloning of the amplified DNA fragments in *Escherichia coli* JM109. DNA was sequenced on both strands with an ABI Prism 310 genetic analyzer (Applied Biosystems). The nucleotide (nt) and amino acid sequences were analyzed by the GENETYX-WIN software package (Software Development, Tokyo, Japan). Multiple amino acid sequences were aligned with the ClustalW program. An unrooted phylogenetic tree was constructed from the aligned sequences by the neighbor-joining method using the TREECON software package as previously described (13).

Construction of a β-xylosidase-specific DNA probe Database searches with N-terminal and internal amino acid sequences determined for the purified enzyme (see Results) revealed that the enzyme was a GH family-3 β-xylosidase. Degenerate oligonucleotides were designed and synthesized according to the N-terminal sequence TAQGGF, the internal sequence VLLKNDG, and a conserved sequence WGRGQET of the GH family-3 β-xylosidases: primer 1 (forward; 5′-ACNGCNCARGGNGGNTT-3′), primer 2 (reverse; 5′-CCRTCRTTYTTNAGNAGNAC-3′), and primer 3 (forward; 5′-TGGGGYMG-HGGYCARGARAC-3′), respectively (H represents A, C, or T; M represents A or C; R represents A or G; Y represents C or T; and N represents any nt). Nested PCR was carried out with each primer pair using *A. pullulans* genomic DNA as the template. Primers 1 and 2 were used in the first-round PCR. The PCR-amplified fragment was separated by electrophoresis on a 2.0% (w/v) agarose gel and purified from the gel using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). The purified DNA fragment served as the template for the second-round PCR using primers 2 and 3. The amplified DNA fragment was cloned into the pCR2.1 plasmid and verified by sequencing as a 716-bp internal fragment of the coding region of the potential β-xylosidase gene *xylI*. The purified PCR product was consequently labeled with digoxigenin (DIG) using the random-primed DNA-labeling and detection kit (Roche). The DIG-labeled 716-bp fragment was used as a *xylI*-specific hybridization probe.

Construction and screening of an *A. pullulans* genomic library Hybridization data (see Results) suggested the presence of the β-xylosidase gene *xylI* on a 6.0-kbp *Eco*RI–*Xba*I fragment. Therefore, *Eco*RI–*Xba*I digests of genomic DNA from *A. pullulans* were fractionated by electrophoresis on a 0.8% (w/v) agarose gel. The gel segment containing approximately 6.0-kbp DNA fragments was excised. The DNA fragments were eluted from the gel and ligated into the *Eco*RI–*Xba*I sites of the pUC18 plasmid. *E. coli* JM109 cells were transformed with the ligation mixture to construct the genomic library that was enriched for the *xylI* gene. Transformant colonies were transferred to Hybond-N^+ nylon membranes (GE Healthcare) and screened for hybridization with the *xylI*-specific DIG-labeled DNA probe.

Mapping of 5′ and 3′ ends of β-xylosidase mRNA Mycelia were harvested from 48-h grown cultures by filtration. Total RNA was isolated from the mycelia with an ISOGEN RNA isolation kit (Wako). The 5′ and 3′ ends of β-xylosidase mRNA were determined by the nested PCR using a GeneRacer kit (Invitrogen) for rapid amplification of 5′ and 3′ cDNA ends (RACE). First-strand cDNA was synthesized from total RNA sample with reverse transcriptase using the oligo(dT) primer provided in the kit. This was used as the template for the first-round PCR. For 5′ RACE, GeneRacer 5′ Primer (forward) and primer 4 (reverse; 5′-CTCGACGCTGATGTCAACACCACCA-3′ complementary to nt 1570 to 1594 relative to the A of the ATG start codon; see Fig. 2) were used in the first-round PCR. The amplified fragment served as the template for the second-round PCR in which GeneRacer 5′ Nested Primer (forward) and primer 5 (reverse; 5′-TGATGATATCGGCCTTCTCAGCAGCA-3′ complementary to nt 1538 to 1562) were used. For 3′ RACE, primer 6 (forward; 5′-CTCAACGCTGGTACTGATCTTGACT-3′ corresponding to nt 1061 to 1085) and GeneRacer 3′ Primer (reverse) were used in the first-round PCR. The amplified fragment served as the template for the second-round PCR in which primer 7 (forward; 5′-GTACCTACTACCAGCACCACCTTC-3′ corresponding to nt 1089 to 1112) and GeneRacer 3′ Nested Primer (reverse) were used. The 5′ and 3′ RACE products were cloned into the pCR2.1 plasmid and sequenced.

Expression of *xylI* cDNA in *P. pastoris* First-strand cDNA was used as the template to amplify the entire *xylI* open reading frame (ORF) with primer 8 (forward; 5′-CGGAATTCACCATGGCTGCTCTACTTTCTC-3′ corresponding to nt −3 to19) and primer 9 (reverse; 5′-ATAAGAATGCGGCCGCTAAGATCCAGGGGCAAG-3′ complementary to nt 2507–2524). 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 *xylI* cDNA fragment was digested with *Eco*RI and *Not*I and cloned into the *Eco*RI–*Not*I sites of the integrative expression vector pPIC3.5 (Invitrogen), yielding pXYL206. pXYL206 was linearized by digestion with *Sac*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. One of the transformants was randomly selected and grown in buffered methanol complex medium (pH 6.0) at 30 °C for 5 days as previously described (14). The cells were separated by centrifugation at 10,000×*g* for 10 min. The supernatant was assayed for β-xylosidase activity as described above.

Nucleotide sequence accession number The nt sequence of the 5199-bp region containing the *xylI* gene will appear in the DDBJ/EMBL/GenBank nt sequence databases with the accession number AB531130.

RESULTS

Purification of a cell-associated β-xylosidase Preliminary experiments showed that only a weak β-xylosidase activity (0.44 U/ml) was present in the culture filtrate of *A. pullulans* strain ATCC 20524 grown on oat spelt xylan and that the enzyme activity was predominantly associated with mycelia. The purification protocol described in Materials and Methods afforded a 60.0-fold increase in specific activity of the β-xylosidase from the cell extract with a yield of 16.7% of the initial activity (Table 1). The purified enzyme had a specific activity of 288 U/mg. The enzyme was homogeneous as judged by SDS–PAGE, which showed an apparent M_r of 88.5 kDa (Fig. 1). The M_r decreased to 83.2 kDa after *N*-glycosidase treatment, as determined by SDS–PAGE. The 5-kDa