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Purification, Characterization, and cDNA Cloning of Xylanase from Fungus *Trichoderma* Strain SY

MIN, SHIN YOUNG, BONG GYU KIM, CHAN LEE[1], HOR-GIL HUR[2], AND JOONG-HOON AHN*

Department of Forest and Environmental Science & Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea
[1]Department of Food Science, Chungang University, Ansung, Korea
[2]Department of Environmental Sience and Engineering, Kwangju Institute of Science and Technology, Kwangju, Korea

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Abstract A xylanase-producing *Trichoderma* strain was isolated from soil. Xylanase from *Trichoderma* strain SY was purified 27-fold to an apparent homogeneity, with a 17.4% yield. The optimum pH and temperature were determined to be 5.5 and 50°C, respectively, and its molecular weight was 21-kDa by SDS-PAGE. The corresponding gene, named *xyl*, was cloned by RT-PCR. DNA blot analysis of *xyl* showed that this gene is present as a single copy. The amino acid sequence of the Xyl protein showed similarity to those of other xylanases derived from various fungi. mRNA of *xyl* was highly expressed when this fungus was grown on cellulose or xylan as a sole carbon source, but undetectable when grown on sucrose. Extracts of *Escherichia coli* cells expressing *xyl* were found to have xylanase activity. It was confirmed that *xyl* from this isolate encodes xylanase.

Key words: *Trichoderma*, xylanase

D-xylan is a major constituent of hemicellulose, which consists of 20-35% of the total dry weight in hardwood and annual plants [7]. The basic structure of xylan is a β-D-(1→4)-linked xylopyranosyl residue with a few branch points [16]. The major backbone carries relatively short side chains with variable lengths. Due to the abundance and the structural heterogeneity of xylans, xylan-degrading enzymes are diverse. Typical xylan-degrading enzymes are endo-β-xylanases which attack the main chain of xylans, and xylosidases which hydrolyze xylooligosaccharides to D-xylose. These two enzymes are produced from many bacteria and fungi [7, 13, 15, 16, 20]. Most xylanases originating from bacteria or fungi are single subunit proteins with molecular weights of 8-145 kDa. Much effort has been made to use these xylanases in biomass conversion [12, 21] and pulp bleaching [17, 18, 19, 26]. On the other hand, xylanases from plant pathogenic microorganisms have been considered as a major virulence factor [4, 27].

Filamentous fungi are more attractive xylanase producers than bacteria or yeast, because they excrete the enzymes into medium to a high level [16]. To understand the hemicellulose degradation by fungal enzymes and evaluate its feasibility for the biomass conversion, a fungal strain of the genus *Trichoderma*, a well-known xylanase producer, was isolated from soil, and a xylanase from this fungus was purified and the corresponding gene was cloned.

*Corresponding author
Phone: 82-2-450-3764; Fax: 82-2-456-7183;
E-mail: jhahn@konkuk.ac.kr

MATERIALS AND METHODS

Fungal Strain and Culture Conditions

The fungus that was used in this study was isolated from soil sampled at the Mukab mountain (Kwangju, Kyoungki-do, Korea). The fungus was identified based on several mycological traits [6]. Sucrose, xylan (Birchwood xylan, Sigma, St. Louis, MO, U.S.A.), oat bran (Quaker Oats Co., Illinois, U.S.A.), and cellulose (C8002, Sigma, St Louis, MO, U.S.A.) were tested as carbon sources. For normal xylanase production, the modified Fries medium [25] supplemented with 1% (w/v) cellulose was used [25], and the fungus was grown in a still culture at 25°C for 9 days.

Enzyme Purification and Analysis

A nine-day old liquid culture was filtered through Whatman No. 1 filter paper. The filtrate was concentrated by ultrafiltration using a 10 kD molecular weight cutoff ultrafiltration membrane. The concentrate was dialyzed against 10 mM sodium acetate buffer (pH 4.0) and loaded onto a CM-cellulose column (2 cm×10 cm) equilibrated with 10 mM sodium acetate (pH 4.0). The protein was eluted with a 10 mM sodium acetate buffer/1.0 M KCl with a linear gradient at a flow rate of 1 ml/min. The fractions that

contained xylanase were collected and concentrated, and loaded onto a Sephadex G-75 FPLC column (Pharmarcia, U.S.A.). The protein was eluted with 25 mM sodium acetate (pH 5.0) containing 0.15 M KCl.

SDS-polyacrylamide gel electrophoresis was carried out in 12.5% (w/v) homogeneous polyacrylamide gel with 5% (w/v) stacking gel [9].

The optimum pH of the purified xylanase was determined with 50 mM buffers composed of sodium citrate (pH 3.0–4.0), potassium acetate (pH 4.5–5.5), potassium phospate (pH 6.0–8.0), and Tris/HCl (pH 8.5–10.0). The optimum temperature was determined at temperatures ranging from 25°C to 70°C.

Xylanase activity was measured by the increase in reducing groups, using birchwood xylan as a substrate. One unit of xylanase was defined as the amount of enzyme that releases 1 μmol of xylose in 1 min at 37°C. The amount of protein was measured by the Bradford method [2].

Molecular Cloning and Expression of Xylanase Gene

The xylanase gene (*xyl*) from *Trichoderma* strain SY was isolated by means of polymerase chain reactions. PCR primers were designed based on the amino acid sequences QAIGPGTG which were experimentally determined from the purified protein, and YQIVAVE, which is an internal sequence highly conserved among related fungal β-1,4-endo xylanases. The PCR primers were CARGCNATHCCNGGNAC (SY1) and TGNACNGCNACDATYTGRTA (SY2; N=A, G, T, C; D=A, G, T; H=A, C, T; Y=C, T; R=A, G). Reverse transcription-polymerase chain reaction (RT-PCR) was carried out with cDNA synthesized from total RNA obtained from mycelium grown on liquid medium, which contained cellulose as a carbon source, under the following conditions: 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 48°C, and 1 min amplification at 72°C. The amplified fragment was subcloned into pGEM-T easy vector (Promega, U.S.A.), and sequenced. The nucleotide sequence of the full length cDNA clone was obtained by 5' & 3' rapid amplification of cDNA end (RACE) [5] using GACGGCAGCGTCTACGACAT and CAGCCGTAGACGGAGAGGTA for 3' and 5' RACE, respectively. Reverse transcription PCR (RT-PCR) was carried out as described [1]. Other nucleic acid manipulations were done as described in Sambrook *et al.* [22].

To construct the expression vector of *xyl*, the full length cDNA of *xyl* was amplified by PCR and subcloned into pGEM-T easy vector. The resulting plasmind (pSY1) was digested with *Eco*RI and *Spe*I, and subcloned into the corresponding sites of pBluscript SK (Stratgene, U.S.A.) to make plasmid pSY2. The *Eco*RI/*Not*I fragment from pSY2 was subcloned into the corresponding sites of pGEX 5X-3 (AmershamPharmacia, U.S.A.). The resulting vector was named pXylexp. To induce xylanase production, the *E. coli* transformant was grown in a LB/ampicilin medium at 37°C. When absorbance at 600 nm reached 0.6, IPTG was added at the final concentration of 0.1 mM, and the transformant was grown for 3 more hours at 25°C. Cells were harvested, resuspended in PBS solution (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.2), and lysed by sonification. The supernatant was used for a xylanase assay and Western blot.

RESULTS AND DISCUSSION

Optimization of Xylanase Production from a *Trichoderma* strain SY

A xylanase-producing fungal strain was discovered by screening two-hundred soil samples. This strain was identified as the genus *Trichoderma*, according to its morphological characteristics as outlined in Games and Bissetts [6] and was named *Trichoderma* strain SY. This strain had the following morphological characteristics; Hyphae of the fungus was initially more or less hyaline and eventually became whitish-green with tufted coidial areas in blue-green shades. Teleomorph was not be detected. Septum of spore was observed. One sterigmata had one conidium whose size was about 8 μm. The shape of conidia was globose or subglobose and rough-walled, and its size was 3.5–4 mm. Based on these characteristics, this fungus was identified to be *Trichoderma* sp. and named *Trichoderma* strain SY.

No xylanase activity was detected in the culture filtrates of *Trichoderma* strain SY when it was grown on sucrose medium. The repression of xylanase production by glucose or sucrose has been observed in the filamentous fungi as well as bacteria [8, 14, 23]. This is known as catabolite repression mediated by a gene, *Crea*. When a fungus grows in a medium containing glucose or sucrose, the protein (Crea) encoded by *Crea* inhibits the transcription of the glucose-repressed genes such as xylanase. This catabolite repression is abolished by phosphorylation of Crea by the Snf1 protein [3, 16, 24].

When cellulose was used as a carbon source, xylanase activity was 0.22 U/mg protein. In the cases of oat meal or xylan, xylanase activity was 0.15 and 0.32 U/mg protein, respectively. In bacteria and fungi, xylanase production has been shown to be inducible, and the mechanism of induction is complex; an inducer for the xylanase production in one species may be an inhibitor in other species [11]. The xylanase in this study was induced by cellulose as effectively as by xylan.

Purification and Properties of Xylanase

The xylanase was purified by ultrafiltration, CM-cellulose chromatography, and gel filtration. The purification scheme is summarized in Table 1. Xylanase was purified 27-fold with 17.4% final yield. The xylanase protein appeared as a

Table 1. Purification of xylanase from a culture filtrate of *Trichoderma* strain SY.

	Total protein (mg)	Total xylanase activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Culture filtrate	24	5.17	0.22	100	1
Ultrafiltration retentate	14.2	4.12	0.29	80	1.3
Dialysate	11.1	3.89	0.35	75	1.6
CM-cellulose chromatography	0.43	1.81	4.21	35	19.1
Sephadex G-75 FPLC	0.19	0.90	6	17.4	27

single 21-kDa band on SDS-PAGE (Fig. 1). There are various kinds of xylanases from different sources and they are classified into families 10 and 11 glycosyl hydrolases by hydrophobic cluster analysis (HCA) [10]. The xylanases that belong to family 10 (Family F) have a high molecular weight and xylanases in family 11 (Family G) have low molecular weight. From the molecular weight of the purified xylanase, it is likely that the purified xylanase belongs to family 11 glycosyl hydrolase.

The purified xylanase was most active at pH 5.5, but at least 60% of the activity was observed at pH 5.0. Also, the enzyme had optimal activity at 50°C. About 80% of the activity at 50°C was detected at 35°C and 55°C. The purified xylanase was digested with trypsin and the internal peptide sequence was determined to be QAIGPGTG.

Isolation and Characterization of Xylanase Gene

To study the regulation of xylanase production at molecular level, the gene designated *xyl* was cloned by PCR. A 500-bp fragment was amplified and the sequence of PCR product showed a high similarity with other fungal xylanases. To obtain the full length clone of *xyl*, 5' & 3' RACE was performed. The full length clone was about 750 bp. The DNA sequence of *xyl* contained an open reading frame of 660 bp (Fig. 2). It was predicted to encode a protein with a molecular mass of 23.5-kDa, pI of 6.82, and to have a signal cleavage site between amino acids 19 and 20. The molecular weight of the protein after cleavage of the signal peptide was 21.9-kD, which is consistent with the molecular mass of purified xylanase (Fig. 1). Blast analysis indicated that the primary amino acid sequence of *xyl* was highly similar to that of an endo β-1,4-xylanase

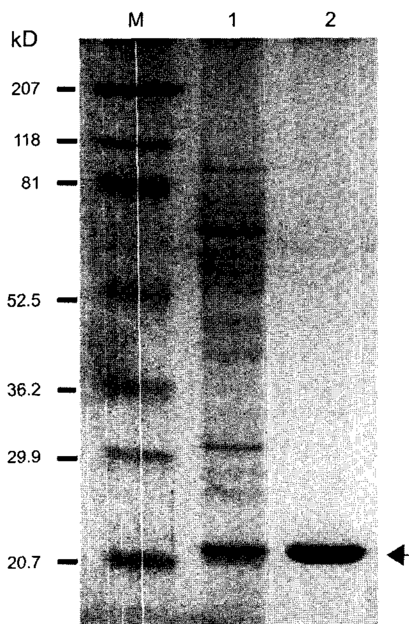

Fig. 1. SDS-PAGE analysis of purified xylanase from *Trichoderma* strain SY.

Lane 1: crude culture filtrate, Lane 2: the purified xylanase. The purified xylanase is indicated as ←.

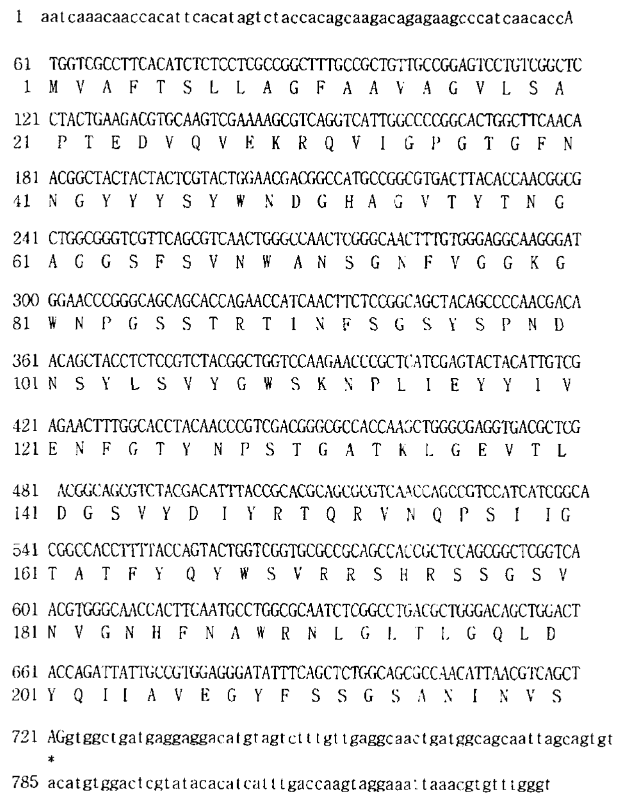

Fig. 2. DNA and the predicted amino acid sequence of the *xyl* from *Trichoderma* strain SY.

Amino acids are shown below the codons. The GenBank accessible number is AY156910.

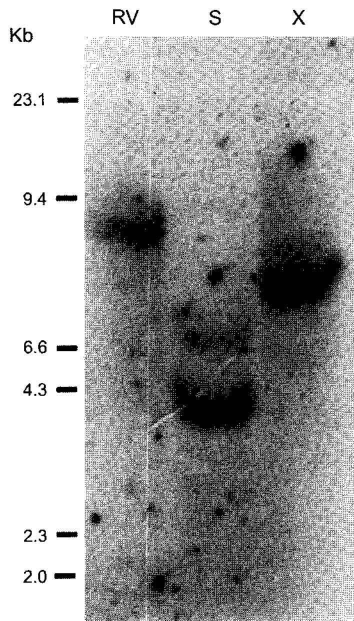

Fig. 3. DNA blot of *Trichoderma* strain SY. Genomic DNA probed with *xyl*.
The DNA was cut with *Eco*RV (**RV**), *Sal*I (**S**), or *Xba*I (X).

from other fungi, especially the species of *Trichoderma*. The protein with the most overall similarity (83% identity) was an endo-β-1,4-xylanase from *T. viride*.

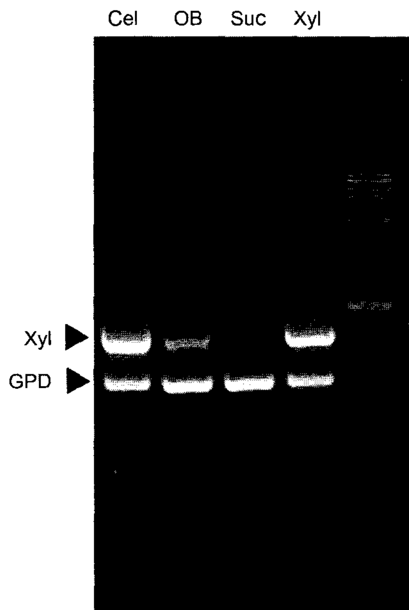

Fig. 4. Expression of *xyl* in *Trichoderma* strain SY.
cDNA was synthesized with total RNA from *Trichoderma* strain SY grown on cellulose (Cel), oat meal (OB), sucrose (Suc), or xylan (Xyl) as a carbon source. PCR was performed with two sets of primers: the first set was specific for the glyceraldehyde 3-phosphate (GPD) gene as a control and the second set was specific for *xyl* of *Trichoderma* strain SY.

Genomic DNA analysis showed that *xyl* was present as a single copy in *Trichoderma* strain SY (Fig. 3). *xyl* was expressed at a higher level on cellulose and xylan (Fig. 4). In oat meal, its expression was decreased, and *xyl* transcripts were hardly detected on sucrose, suggesting that *xyl* was induced by complex sugars and repressed by glucose or sucrose. The repression by glucose has also been observed in other xylanases from several microorganisms as well as cell wall degrading enzymes from other fungi [16, 24], in agreement with that of the xylanase activity assay with different carbon sources.

Expression of *xyl* in *E. coli*

To verify that the *xyl* encodes a xylanase, *xyl* was expressed in *E. coli*. The expressed proteins were analyzed by SDS-PAGE. The molecular weight of the expressed protein was about 47-kDa, which corresponded to the sum of that of the xylanase (21-kDa) and the N-terminal fusion protein containing glutathione S-transferase (GST; 26-kD). Western blot analysis of the above total proteins with anti-GST antibody also indicated the strong band to be the induced xylanase (Fig. 5). The soluble protein from an extract of *E. coli* harboring the plasmid pXylexp or the vector pGEX 5X-3 was tested for xylansase activity. The soluble protein from *E. coli* producing *xyl* exhibited xylanase activity (0.35 U/mg protein), whereas *E. coli* extracts from the control culture harboring the expression vector without the insert had no xylanase activity. These results confirm that *xyl* encodes a xylanase.

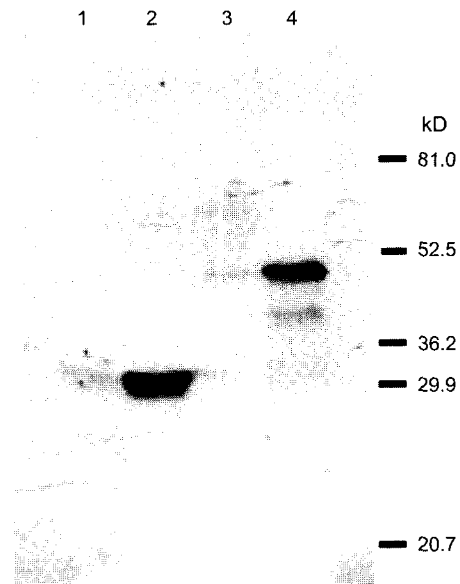

Fig. 5. Western blot analysis of the xylanase fusion protein in *E. coli*.
Lane 1; soluble protein of *E. coli* DH5α harboring pGEX 5X-3 without induction, Lane 2; soluble protein of *E. coli* DH5α harboring pGEX 5X-3 with induction, Lane 3; soluble protein of *E. coli* DH5α harboring pXylexp without induction, Lane 4; soluble protein of *E. coli* DH5α harboring pXylexp with induction.

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