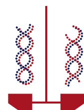
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Characterization of a recombinant bifunctional xylosidase/arabinofuranosidase from *Phanerochaete chrysosporium*

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A bifunctional xylosidase/arabinofuranosidase gene (*PcXyl*) was cloned from the cDNA library of *Phanerochaete chrysosporium* and further expressed in *Pichia pastoris*. Enzymatic assay indicated that *P. pastoris* produced rPcXyl at a level of 26,141 U l^{-1}. The xylosidase and arabinofuranosidase activities of rPcXyl were maximized, respectively, at pHs of 5.0 and 5.5 and temperatures of 45°C and 50°C. SDS-PAGE revealed a single band of purified rPcXyl of 83 kDa. Cu^{2+} and Zn^{2+} completely inhibited the enzyme activity of rPcXyl. The enzyme activity of rPcXyl was increased 151%, 126% and 123%, respectively, in the presence of glucose, xylose and arabinose at concentrations of 5 mM. rPcXyl hydrolyzed xylobiose to xylose and xylotriose to xylose and xylobiose, indicating rPcXyl acts as an *exo*-type enzyme. Additionally, rPcXyl enhanced xylose release from xylan substrates in synergy with rPcXynC.

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[**Key words:** *Phanerochaete chrysosporium*; *Pichia pastoris*; Xylosidase; Arabinofuranosidase; Xylan; Xylose]

Hemicellulose comprises a complex polysaccharide structure mainly composed of pentose (xylose, arabinose) and hexose (glucose, mannose) sugars, the most abundant of which is xylose. For complete hydrolysis of hemicellulose, the synergetic actions of numerous enzymes are required. These enzymes include endo-xylanases (E.C 3.2.1.8), β-xylosidases (E.C 3.2.1.37), endo-arabinan ases (E.C.3.2.1.99), α-L-arabinofuranosidases (E.C 3.2.1.55), α-glucuronidases (E.C 3.2.1.139), endo-mannanases (E.C 3.2.1.78), β-man osidases (3.2.1.25), α-galactosidases (E.C 3.2.1.22), β-glucosidases (E.C 3.2.1.21) and esterases (E.C.3.1.1.-). Among these, xylanases and β-xylosidases are the key enzymes responsible for the hydrolysis of xylan, the major component of hemicelluloses. Xylanases hydrolyze the internal β-1,4 linkage of the main chain of xylan, yielding short xylo-oligomers, which β-xylosidases cleave to release single units of xylose (1,2).

β-Xylosidases are classified into eight families of glycoside hydrolases based on their amino acid sequence similarities and biochemical properties. Enzymes from families 3, 30, 39, 52, 54, 116, and 120 catalyze the hydrolysis of xylo-oligomers via a retaining mechanism, while enzymes from family 43, however, perform hydrolysis by inverting the anomeric configuration of xylo-oligomers (2,3). β-Xylosidases are essential enzymes of xylanolytic systems in microorganisms, along with xylanases, prevent end-product inhibition of hydrolysis enzymes by xylo-oligomers. Interestingly, numerous β-xylosidases isolated from microbial systems have been reported to exhibit bifunctional β-xylosidase/α-L-arabinofuranosidase activity, including those from a compost starter mixture metagenome (4), *Paenibacillus woosongensis* (5), *Thermoanaerobacter ethanolicus* (6), and *Penicillium purpurogenum* (7). The bifunctional activities of β-xylosidases are due to their spatial similarities with D-xylopyranose and L-arabinofuranose, particularly their glycosidic bonds and hydroxyl groups (1). Xylosidases and arabinofuranosidases are important enzymes in biomass saccharification. These enzymes not only increase the release of reducing sugars, but also prevent the inhibition of other hydrolysis enzymes in synergistic action with xylanase or cellulase (8–10). Thus, these enzymes are of great potential in biotechnology and industrial applications due to their synergetic activities.

Xylose is an abundant sugar in hemicelluloses. Moreover, xylose is found in agricultural and agro-industrial materials, such as barley straw, corn stalks, corn stover, rice straw, and wheat straw, comprising 15%–35% of each plant's dry weight (11). Xylose can be utilized to produce bioethanol or valuable compounds used in food, pharmaceutical and industry (12,13). To produce xylose from biomass sources, numerous methods have been developed including chemical and enzymatic methods (11). Enzymatic treatments are preferred due to their environment friendliness, recyclability, and effectiveness.

In this study, we cloned a putative β-xylosidase gene from the cDNA library of *Phanerochaete chrysosporium*, which was then expressed in *Pichia pastoris*. The biochemical characterization of the

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resultant enzyme was investigated and found to exhibit bifunctional β-xylosidase/α-L-arabinofuranosidase activity.

MATERIALS AND METHODS

Strains, plasmids and media The white rot fungus *P. chrysosporium* BKM-F-1767 was obtained from the Korean Collection for Type Culture and maintained in medium as described by Tien and Kirk (14). Gene cloning was carried out using a pGEM-T Easy vector (Promega, Madison, WI, USA). The recombinant vector was transferred into *Escherichia coli* Top10, which was subsequently screened on LB agar plate containing 50 μg ml^{-1} ampicillin, supplemented with IPTG and X-Gal. *P. pastoris* GS115 (*his4*) and vectors pPICZ and pPICZα were purchased from Invitrogen (Carlsbad, CA, USA) and utilized as the expression host and vectors, respectively. The *PcXyl* gene was constructed with pPICZC or pPICZαC. The resulting construct was then introduced into *E. coli* Top10 and selected using low-salt LB medium containing 25 μg ml^{-1} of Zeocin. Recombinant β-xylosidase (rPcXyl) expression was studied by inoculating recombinant *P. pastoris* in YP medium (1% yeast extract, and 2% peptone) containing 1% methanol.

cDNA library Total mRNA was isolated from *P. chrysosporium* mycelia using the Oligotex mRNA Mini kit (Qiagen, Valencia, CA, USA). The cDNA library was synthesized using the SMARTer PCR cDNA Synthesis kit following the manufacturer's instructions (Clontech, Mountain View, CA, USA).

Gene manipulation The nucleotide sequence of the putative cDNA of *PcXyl* was obtained from the RP78 genome database (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html). The full-length cDNA of *PcXyl* was amplified using the forward PcXyl-F 5′-GAATTCATGCACCGTATTGCGAGGGC-3′ and reverse PcXyl-R 5′-GGGCCCATAAACGTTCTCTACAGGTT-3′ primers. Additionally, *PcXyl* cDNA without secretion signal was generated with the forward PcXylα-F and reverse PcXylα-R primers of 5′-GAATTCGGTCGTGTCGCTCACTCGAA-3′ and 5′-CCGCGGAATAAACGTTCTCTACAGGT-3′, respectively, resulting in an amplicon deemed *PcXylα*. The PCR conditions of 94°C for 1 min, 94°C for 30 s, 62°C for 30 s, 72°C for 2 min (for 30 cycles), and 72°C for 10 min were employed for PCR amplification using *Pfu* DNA polymerase (Solgent, Daejeon, South Korea). The expected PCR products were excised and purified from a 0.8% agarose gel, after which an A-tail was added by *Taq* DNA polymerase (Takara, Shiga, Japan) at 72°C for 30 min, followed by ligation with pGEM-T Easy Vector. *E. coli* transformants were screened by colony PCR with PcXyl-F and PcXyl-R or PcXylα-F and PcXylα-R primers. Three recombinant plasmids were randomly isolated and sequenced. The *PcXyl*-pGEM-T gene was excised using *Eco*RI and *Apa*I, whereas PcXylα-pGEM-T was incubated with *Eco*RI and *Sac*II. The *PcXyl* and *PcXylα* genes were separated and further purified on a 0.8% agarose gel. The purified *PcXyl* and *PcXylα* genes were then inserted into the pPICZA and pPICZαA vectors, respectively, resulting in pPICZA-*PcXyl* and pPICZαA-*PcXylα*. A sequence analysis was performed on pPICZA-*PcXyl* or pPICZαA-*PcXylα* using AOX1 primer according to the manufacturer's instructions.

The pPICZA-*PcXyl* and pPICZαA-*PcXylα* plasmids were linearized using *Pme*I, after which one of the two was then transformed into *P. pastoris* GS115 using an electroporation method, as recommended by the manufacturer (Bio-Rad, Hercules, CA, USA). Transformed cells were selected on YPD agar plates containing 100 μg ml^{-1} of zeocin and 1 M sorbitol at 30°C, until colonies were observed (2–3 days). The integration of *PcXyl* or *PcXylα* into the *P. pastoris* genome was confirmed by PCR using AOX1 primers.

Enzyme induction The qualification expression of twenty positive *P. pastoris* transformants were examined by dot blot analysis using His-tag antibody as described by Vasu et al. (15). The highest secreted *P. pastoris* transformants were grown in 5 ml YPD medium at 30°C and 200 rpm for 24 h, and then transferred to 50 ml of fresh YP medium containing 1% glycerol in a shaking incubator at 30°C and 180 rpm overnight. Enzyme induction was carried out in 100 ml of YP medium at an initial cell optical density of 1.0, supplemented with 1% methanol for every 24 h for 7 days. One milliliter of cultivation fluid was collected every 24 h and centrifuged for 5 min at 15,000 rpm, after which enzyme activity was measured.

Enzyme activity determination Xylosidase activity was determined by measuring the release of *p*-nitrophenol (pNP) from *p*-nitrophenyl-β-D-xylopyranoside (pNPβX) (Sigma, St. Louis, MO, USA) as described by Kim and Yoon (5). Briefly, 90 μl of supernatant was mixed with 100 μl of 100 mM sodium acetate, pH 5.0, at 40°C. The reaction was initiated by adding 10 μl of 50 mM pNPX in ethanol and incubating for 10 min at 40°C. The reaction was terminated by adding 1 ml of 1 M sodium carbonate. The amount of pNP released was determined at 410 nm wavelength using a TCC-240A UV spectrophotometer (Shimadzu, Kyoto, Japan). A standard curve was generated using pNP as a substrate, and the absorbance was converted into moles of pNP released. One unit of xylosidase activity was defined as the release of 1 nmol per minute of pNP under experimental conditions.

Enzyme purification and deglycosylation Cell-free supernatant was collected by centrifugation at 2000 rpm for 5 min, filtered through 0.45 μm filters, and then mixed with 10× binding buffer (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole; pH 7.4). Enzyme was purified using a Ni^{2+} His-tag column (Histrap-GE Healthcare, Picataway, NJ, USA) with an ÄKTA fast protein liquid chromatography purification system. Enzyme was eluted with an elution buffer (20 mM sodium phosphate, 0.5 M NaCl and 500 mM imidazole; pH 7.4) and collected into 15 ml conical tubes. Purified enzyme (rPcXyl) was dialyzed with a cellulose dialysis tubing membrane (Sigma, St. Louis, MO, USA) against distilled water at 4°C overnight. Protein concentration was estimated by the Bradford method using a Thermo Scientific Protein Assay kit (Rockford, IL, USA), taking serum albumin as the standard.

The glycosylation of rPcXyl was analyzed using Endoglycosidase H. Approximately 5 μg of purified rPcXyl were incubated with 500 unit of enzyme for 30 min, 37°C. The molecular mass of purified rPcXyl and deglycosylated rPcXyl were determined by SDS-PAGE.

Effect of pH, temperature, metal ions and sugars on enzyme activity The effect of pH on rPcXyl activity was determined by assaying for enzyme activity at 40°C at different pH values of 3.0–6.0 using 50 mM sodium acetate and 7.0–8.0 using 50 mM sodium phosphate buffer. The reaction mixture was incubated at the specified temperature for 30 min, after which 10 μl of purified enzyme was added for 10 min. The amount of pNP released was then measured as described above. The optimal temperature for rPcXyl activity was examined at temperatures of 30–60°C at the optimal pH.

The effect of metal ions (Mn^{2+}, K^{+}, Ca^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, and Ni^{2+}) on rPcXyl activity was investigated to further characterize the enzyme. rPcXyl was incubated with 10 mM each of metal ion at the optimal pH and temperature. The retained activity of rPcXyl was compared with the enzyme activity thereof in a non-metal reaction.

Additionally, the effect of sugars (glucose, xylose and arabinose (Sigma, St. Louis, MO, USA)) on rPcXyl activity was also studied. To do so, rPcXyl was incubated for 1 h at optimal pH and temperature with 5 mM, 10 mM, 50 mM, or 100 mM of each sugar. The retained activity of rPcXyl was measured and compared to that in the absence of sugar.

Substrate specificity The substrate specificities of rPcXyl were investigated using sugar beet arabinan, debranched arabinan, wheat arabinoxylan (Megazyme, Bray, Co. Wicklow, Ireland), birchwood xylan, beechwood xylan, as well as *p*-nitro phenyl-β-D-glucopyranoside (pNPG), *p*-nitrophenyl-α-D-xylopyranoside (pNPαX), *p*-nitrophenyl-α-L-arabinofuranoside (pNPA), and *p*-nitrophenyl-α-D-xylopyranoside (pNPαX) (Sigma, St. Louis, MO, USA). pNPG, pNPαX, and pNPA hydrolysis were carried out in the same manner performed in the pNPβX assay. The sugars released by hydrolysis reactions of sugar beet arabinan, debranched arabinan, wheat arabinoxylan, birchwood xylan, and beechwood xylan were measured by the 3,5-dinitrosalicylic acid (DNSA) method (16). One unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmol reducing sugar per minute.

The competitive inhibition of α-L-arabinofuranosidase substrate on xylosidase activity of rPcXyl was performed by adding pNPA into 100 μl of 50 mM sodium acetate pH 5.0 reaction containing 5 mM of pNPβX. The reaction was carried out for 10 min at 45°C, after which the amount of pNP released was determined as described above.

Hydrolysis of xylo-oligomers and xylan substrates in synergistic action with xylanase The mode of action of rPcXyl was examined using xylo-oligomers (xylobiose, xylotriose, and xylopentaose), while the synergistic action of rPcXyl and rPcXynC (17) were evaluated using birchwood and beechwood xylans. Hydrolytic products of xylo-oligomers and xylan substrates were analyzed by thin layer chromatography (TLC) using chloroform/acetic acid/H_2O (6:7:1) as a mobile phase. Reaction products were visualized by spraying TLC plates with sulfuric acid/ethanol (5:95, v/v) containing 1 mg/ml of orcinol, followed by baking at 110°C for 5 min. Xylose, xylobiose, xylotriose, and xylopentaose were used as standards.

The synergistic action of rPcXyl on xylan (birchwood xylan, beenchwood xylan and arabinoxylan) was investigated using the rPcXynC. The reducing sugar released by hydrolysis reaction was measured as method described above.

Additionally, the transglycosylation activity of rPcXyl was determined using pNPβX and various alcohol receptors (ethanol, methanol, 2-propanol) as described by Shao et al. (3). The hydrolysis products were separated and visualized by TLC assay as described above.

RESULTS

Cloning and sequence analysis of *PcXyl* Previously, we reported the successful cloning and expression of a *P. chrysosporium* xylanase (17). In order to degrade hemicellulose substrates, we investigated other *P. chrysosporium* hemicellulases for use in enhancing the synergistic action of xylanase. Based on the *P. chrysosporium* RP78 genome database, we found a putative family 43 glycoside hydrolase that comprises a xylosidase conserved domain. The putative xylosidase gene is composed of 10 exons and 9 introns that encode for 598 amino acids with a predicted signal peptide of 26 residues. The predicted cDNA sequence of this putative xylosidase was used as a template to design primers for gene amplification from the total *P. chrysosporium* BKM-F-1767 cDNA library. The PCR amplicon was

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