Cloning and characterization of a novel bifunctional acetyl xylan esterase with carbohydrate binding module from _Phanerochaete chrysosporium_

Nguyen Duc Huy,1,2 Saravanakumar Thiagarajan,1 Dae-Hyuk Kim,4 and Seung-Moon Park1,3,*

Division of Biotechnology, College of Environmental and Bioresource Sciences, Chonbuk National University, 194-5 Madong, Iksan, Jeonbuk 570-752, Republic of Korea,1 Institute of Resources, Environment and Biotechnology, Hue University, Vietnam,2 LED-AgroBio Fusion Technology Research Center, Chonbuk National University, Iksan, Jeonbuk 570-752, Republic of Korea,1 and Department of Molecular Biology, College of Natural Sciences, Chonbuk National University, Jeonju, Jeonbuk 561-756, Republic of Korea4

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The cDNA of acetyl xylan esterase 2 (PcAxe2) gene containing a carbohydrate binding module (CBM) sequence from _Phanerochaete chrysosporium_ was cloned and expressed in _Pichia pastoris_. The recombinant PcAxe2 protein (rPcAxe2) was efficiently produced, reaching a maximum of 1058 U l−1 after 6 days of cultivation. Molecular mass of the rPcAxe2 on SDS-PAGE was approximately 63 kDa under hyperglycosylation. Optimal activity of the purified rPcAxe2 enzyme was observed at pH and temperature of 7.0 and 30–35 °C, respectively. In addition to acetyl xylan esterase activity, rPcAxe2 also exhibited a xylanase activity at an optimum pH and temperature of 5.0 and 80 °C, respectively. The synergistic action of rPcAxe2 with rPcXynC on birchwood xylan, beechwood xylan and wheat arabinoxylan enhanced the total reducing soluble sugar.

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[Key words: Acetyl xylan esterase; _Phanerochaete chrysosporium_; _Pichia pastoris_; Synergisms; Xylanase]

Xylan is a major constituent of hemicelluloses, which are the second most abundant renewable polysaccharides in nature after cellulose. Xylan accounts for 20–30% of the biomass in hardwoods and herbaceous plants, whereas it increases up to 50% in some grass and cereal tissues. Xylan is composed of a β-1,4-xylol backbone with arabinofuranose, glucuronic acid, methylglucuronic acid and acetyl side groups (1,2). Approximately 60–70% of xylose residues in hardwood xylan are substituted with acetyl groups in the O-2 or O-3 position (3).

Xylan degradation consequently requires complex enzymes. These enzymes are classified into two groups based on the nature of the linkages that they cleave. The first group of enzymes is hydrolases, which are involved in xylan hydrolysis through the glycosidic bonds. Xylanase (EC 3.2.1.8) breaks down the β-1,4-backbone of xylan, β-xylolidas (EC 3.2.1.37) cleaves the xylooligosaccharides yielding xylose, and α-D-glucuronidase (EC 3.2.1.139) removes the arabinose and 4-O-methylglucuronic acid substituents. The second group includes enzymes that rupture the ester linkages between xylose units of the xylose polymer and acetic acid (acetyl xylan esterase, EC 3.1.1.72) or between arabinose side-chain residues and phenolic acids (ferulic and p-coumaric acid esterases, EC 3.1.1.73) (4,5). The mechanism of acetyl xylan esterase is to bind to the plant cell wall and remove the acetyl group, subsequently increasing the solubility of xylan and lignin, creating new sites for xylanase or lignocellulase action (6,7).

Acetyl xylan esterases (Axe) are produced by a variety of cellulolytic and hemicellulolytic organisms. Numerous genes encoding for Axe have been cloned and expressed from _Orpinomyces sp._ (8), _Trichoderma reesei_ (3), _Neocallimastix patriciarum_ (9) in _Escherichia coli_, and _Thermobifida fusca_ (10), _Aspergillus fumigatus_ (11), A. awamori (12), _A. oryzae_ (13) in _Pichia pastoris_, respectively.

The white rot fungus _Phanerochaete chrysosporium_ is capable of degrading woody biomass by enzymatic processes. Biomass degradation by this fungus is accomplished through a complex mixture of cellulases, hemicellulases and ligninases. A genome sequence shows that its putative xylan-degrading system involves xylanases, acetyl xylan esterases, α-D-arabinofuranosidases, and glucuronol esterases (α-D-glucuronidase) [Supplementary of Martinez et al. (14)]. These enzymes are also found in the extracellular protein matrix when _P. chrysosporium_ is cultured in cellulose-grown medium.

_P. chrysosporium_ genome sequence shows three putative Axe genes, named Axe1, Axe2, and Axe3 [Supplementary of Martinez et al. (14)]. The Axe1 gene consists of 1017 nucleotides, whereas Axe2 and Axe3 are encoded by 1104 and 624 nucleotides, respectively. Among these genes, the Axe1 and Axe2 were identified in the _P. chrysosporium_ strain BKM-F-1767 by protein secretome database analysis (15,16). However, the functional aspects of these genes are still unknown.

The present work describes the cloning of a cDNA corresponding to the putative Axe2 gene from _P. chrysosporium_ BKM-F-1767. The protein encoded by the gene, hereafter called PcAxe2, carries a carbohydrate binding module (CBM) of fungal type at its N-terminus. Further
bifunctional enzymatic characterization and synergistic action of this enzyme for xyloglucan degradation are also reported.

**MATERIALS AND METHODS**

**Strains, plasmids and medium cultures**
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 (17). E. coli Top10 was cultured in LB agar medium containing 50 μg ml⁻¹ ampicillin with IPTG and X-Gal, for general cloning, and a low-salt LB medium containing 25 μg ml⁻¹ Zeocin for *P. pastoris* genes constructed with electroporation vectors. The heterologous expression host *P. pastoris* X33 was obtained from Invitrogen (Carlsbad, CA, USA). Stock cultures were prepared by an overnight incubation on YPD agar medium (1% yeast extract, 2% peptone, 2% dextrose, and 1.5% agar) at 30°C. *PcAxe2* expression was carried out by inoculating recombinant *P. pastoris* in YM medium (1% yeast extract, and 2% peptone) containing 100 mM sodium phosphate (pH 6.0) and 1% methanol.

The pGEM-T Easy Vector (Promega, Madison, WI, USA) was constructed with the *PcAxe2* polymerase chain reaction (PCR) products for gene sequencing. The vectors pPICZC and pPICZC/n (Invitrogen) were used for expression in *P. pastoris*. Expression of inserts in both vectors is controlled by the methanol-inducible AOX1 promoter.

**Construction and transformation of the recombinant plasmid**
The nucleotide sequence of the *P. chrysosporium* *PcAxe2* was obtained from the *P. chrysosporium* genome database of the DOE Joint Genome Institute (http://genome.jgi-psf.org/Phchr1). The cDNA of *PcAxe2* was excised using *SalI* and *XhoI* for 0.8-kb agarose gel. The purified cDNA was inserted into the pGEM-T Easy Vector, excised using *SalI* and *XhoI*. Then, the cDNA was ligated into the pPICZ vectors using *SalI* and *XhoI*. The resulting construct was digested using *BamHI* and *SacI* for 0.8-kb agarose gel. The purified cDNA was inserted into the pPICZC/n vector, excised using *SalI* and *XhoI*. The resulting construct was digested using *BamHI* and *SacI*

**Expression and production of recombinant *PcAxe2**

Ten *P. pastoris* transformants were cultured in 50 ml YM medium containing 1% methanol to determine extracellular enzyme activity by enzyme assay. To measure *PcAxe2* expression, a single colony was grown in 5 ml YM medium overnight at 30°C and 200 rpm. Then, 5 ml cultures were transferred to 50 ml of fresh YM medium containing 1% glycerol in a shaking incubator at 30°C and 150 rpm for 1 day. The cell pellets were harvested by centrifugation at 2000 rpm for 5 min, and the cell pellet was resuspended in 10 ml YM medium. Then, the suspensions were added slowly to 90 ml of fresh YM medium supplemented with 110 mM sodium phosphate (pH 6.0) until an optical density of 1 was reached. Finally, fresh YM medium was added to make the final volume to 100 ml. To induce *PcAxe2*, 1 ml of 100% methanol was added every 24 h to a final concentration of 1%, for 7 days of culture at 25°C. One milliliter of crude filtrate was collected every 24 h and centrifuged for 5 min at 15,000 rpm, after which enzyme activity was measured.

**Enzyme purification**

For purification of *PcAxe2*, on the second day of culture, cell-free supernatants were collected by centrifugation at 2000 rpm for 5 min, and mixed with 10 ml of 10X binding buffer (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole; pH 7.4). The mixture was applied to a Ni²⁺ His-tag column (HisTrap, GE Healthcare, Piscataway, NJ, USA) using the AKTA fast protein liquid chromatography purification system. Protein was eluted with elution buffer (20 mM sodium phosphate, 500 mM sodium chloride, and 200 mM imidazole; pH 4.5) at a flow rate of 2 ml/min. The fractions collected were dialyzed in 15 ml fractions. All fractions containing purified enzyme were dialyzed in distilled water to remove salt and imidazole overnight at 4°C.

**Cellulose and xylan binding by *PcAxe2**

*PcAxe2* (10 μl) was incubated with 100 mg of microcrystalline cellulose or birchwood xylan (Sigma, St. Louis, MO, USA) in 100 mM sodium phosphate (pH 7.0) at 4°C for 30 min with gentle shaking. The samples were applied on Poly-Prep columns (Bio-Rad, Hercules, CA, USA), and were washed with three columns of buffer. In the final step of binding, the columns were washed with three columns of buffer containing 0.25% bovine serum albumin. Elution of bound enzyme was performed washing with a similar volume of buffer containing 1.3% cellulose or xylose, respectively. Activity in all fractions was measured as described below.

**Deglycosylation and electrophoresis**

*PcAxe2* glycosylation was analyzed using endoglycosidase H and peptide-N-glycosidase F. Approximately 2 μg of purified *PcAxe2* was incubated with 500 units of enzyme for 30 min. After the incubation, the deglycosylated *PcAxe2* was subjected to SDS-PAGE as described by Sambrook and Russell (18).

**Enzymatic assay**

Activity was determined spectrophotometrically as described by Chung et al. (11). Ten microliters of supernatant was mixed with 980 μl of 100 mM sodium phosphate buffer (pH 7.0) at 30°C. The reaction was started by adding 10 μl of 10 mmol p-nitrophenylphosphate to the mixture, which was incubated for 5 min at 30°C. The released p-nitrophenol was determined at a wavelength of 410 nm using a TCC-240A UV spectrophotometer (Shimadzu, Kyoto, Japan). One unit of *Axe* activity was defined as the amount of enzyme required to release 1 mmol of p-nitrophenol per min at 37°C. Protein concentration was estimated by the Bio-Rad method, using the BSA as a standard.

**Scientific Protein Assay Kit** (Rockford, IL, USA) with serum albumin as the standard.

**Xylanase assay**

Activity was assayed by measuring the production of reducing sugar from birchwood xylan (Sigma, St. Louis, MO, USA) using 3,5-dinitrosalicylic acid (DNSA). One unit of enzyme was defined as the release of 1 μmol/min of xylose. Effect of pH temperature, metal ions, detergents and surfactants on acrylamide gel and sodium acetate xylanase activity were determined.

The effect of pH on *PcAxe2* was determined by assaying for enzyme activity at 35°C with different pH levels in the range of 4.5—8.5 using 100 mM sodium phosphate buffer. The optimal temperature was investigated in the range of 10—50°C at the optimal pH. Thermal stability of the *PcAxe2* was determined by incubating the enzyme solution for 1 h at the appropriate temperature (10—50°C). The effect of metal ions (Mg²⁺, K⁺, Ca²⁺, Fe³⁺, Fe⁺, Fe⁴⁺, Cu²⁺, Zn²⁺, and Ni²⁺), inhibitors phenylmethanesulfonyl fluoride (PMSF) and surfactants [Tween20, Tween80, Triton X-100, and sodium dodecyl sulfate (SDS)] on *PcAxe2* activity was investigated. Further characterization of the enzyme, *PcAxe2* was preincubated with the aforementioned chemicals for 1 h at the appropriate temperature, and the residual activity was measured using a standard *Axe* assay. The final concentrations of each metal ion and PMSF were used and 5 and 1 mM, respectively, whereas the concentrations of SDS and surfactant were 0.5% at the time of the preincubation. Effect of temperature and pH on *PcAxe2* activity and xylan-oligosaccharides hydrolysis.

**Xylan enzyme activity**

Xylanase activity was determined by performing the xylanase assay, as described above, at a temperature range of 40—90°C, whereas a pH range of 3.0—8.0 was used to determine the optimum pH.

**Xylose, xylitol or xylopetase (Sigma, St. Louis, MO, USA) at concentrations of 5 mM, 3.3 mM or 2 mM, respectively, were incubated with 1 μl of *PcAxe2* for 200, 150 or 100 μl of 100 mM sodium acetate buffer at pH 5.8. The reaction was terminated by heating at 100°C for 5 min, after which 1 μl of product reactions were taken and analyzed using thin layer chromatography (TLC) on silica gel with chlorform/acetic acid/H₂O (6:7:1) as a mobile phase. Reaction products were visualized by spraying TLC plates with a mixture containing 1 mg of orcinol, followed by baking at 110°C for 5 min. Xylose, xylobiose, xylotriose, and xylopetase were used as standards.

**Peracetic acid biosynthesis**

The perhydrolysis activity of *PcAxe2* was quantitatively measured by estimating the sulphoxide content using high performance liquid chromatography (HPLC) as described by Park (19). Four units of *PcAxe2* were incubated with 1 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 500 mM ethyl acetate and 1 M hydrogen peroxide at 35°C for 30 min. Then, 0.1 ml of the reaction solution was mixed with 0.1 ml of 20 mM methyl p-tolyl sulfoxide (MTS) and 0.8 ml of 60% acetonitrile and incubated again at 35°C for 60 min. Ten milligrams of manganese dioxide was added to the solution and centrifuged at 15,000 rpm for 5 min. The amount of MTS and its oxidizing derivatives, such as methyl p-tolyl sulfoxide and methyl p-tolyl sulphone, were analyzed by HPLC (Shimadzu, Kyoto, Japan) with a reverse-phase C₁₈ column.
RESULTS AND DISCUSSION

Cloning and sequence analysis of \textit{PcAxe2}  
Specific primers were designed to amplify \textit{Axe} genes from the total cDNA of \textit{P. chrysosporium} BKM-F-1767 based on the predicted nucleotide sequences of the \textit{P. chrysosporium} RP78 \textit{Axe1} and \textit{Axe2} genes. PCR products of the predicted \textit{Axe2} gene was obtained and was named as \textit{PcAxe2}. The nucleotide sequence of this cDNA product showed that it consisted of 1104 nucleotides and corresponded to a gene model with the protein model designation 63763 (or Axe2) of version 1.0 of the \textit{P. chrysosporium} RP78 genome sequence. Both shared 98% nucleotide similarity. The deduced amino acid alignment showed 99% identity, whereas the cloned \textit{PcAxe2} had four additional amino acids. The full-length cDNA nucleotide sequence of \textit{PcAxe2} in this study was deposited in GenBank (accession no. JQ031636).

A signal peptide of 16 amino acids was identified using the signal peptide detection tool available on the website http://www.cbs.dtu.dk/services/SignalP/. A consensus motif of the active site is present in the deduced amino acid sequence of the cloned \textit{PcAxe2}.

![Alignment of amino acid sequences](image)

**FIG. 1.** (A) The deduced amino acid sequence alignment of \textit{P. chrysosporium} acetyl xylan esterase 2 (\textit{PcAxe2}) and other Axes from \textit{A. ficuum} (AF331757), \textit{A. niger} (XP_001395572), \textit{A. oryzae} (XM_001826277), \textit{Talaromyces emersonii} (HQ185919), and \textit{A. kawachii} (CAA86314). The fungal carbohydrate binding module (CBM) sequence is underlined. The consensus motif of the active site serine enzyme (Gly-Xaa-Ser-Xaa-Gly) and the Asp294 and His351 conserved catalytic residues are marked with asterisks. The two N-glycosylation sites (Asn-Xaa-Ser/Thr) are boxed. (B) Amino acid sequence alignment of the CBM of \textit{PcAxe2} and others from \textit{P. chrysosporium} endo-1,4-\beta-xylanase A (AAG44992), \textit{Trichoderma reesei} carbohydrate binding module (EGR44948), \textit{Trichoderma virens} esterase (EHK25849), and \textit{Dichomitus squalens} cellobiohydrolase II (EJF66131).
serine enzyme (Gly-Thr-Ser-Ser-Gly) and two N-glycosylation sites (Asn-Asn-Thr and Asn-Ala-Thr) were also found on PcAxe2. The PcAxe2 amino acid sequence was searched using BLAST and aligned by Clustal W version 2.0 software ([http://www.ebi.ac.uk/Tools/msa/clustalw2/](http://www.ebi.ac.uk/Tools/msa/clustalw2/)). As shown in Fig. 1, PcAxe2 revealed a striking level of identity to Axes from A. fuscum (51%), A. niger (52%), A. oryzae (51%), Talaromyces emersonii (53%) and A. kwunghui (50%). Asp294 and His351 of A. oryzae (51%), Talaromyces emersonii (53%), and other fungal esterase classified in the carbohydrate esterase family (Fig. 1B). Interestingly, the catalytic modules of the endo-1,4-β-xylanase A and cellobiohydrolase II do not belong to the family of carbohydrate esterases indicating that the catalytic modules and CBMs may not functionally related, and evolved separately in the gene.

Expression and production of rPcAxe2 The methylotrophic yeast P. pastoris is a useful system to express milligram-to-gram quantities of proteins for both basic laboratory research and industrial purposes. P. pastoris has a low specificity requirement for secretion signal sequence recognition; thus, many recombinant proteins might be expressed in P. pastoris using the native secretion signal peptide. Moreover, the glycosylation system of P. pastoris is less efficient than that of other heterologous expression systems such as S. cerevisiae; therefore, it secretes a native recombinant protein or a less hyperglycosylated form (21,22). Numerous polysaccharide degradation enzymes from P. chrysosporium have been successfully expressed in P. pastoris using the intrinsic signal peptide (13,20,23). In this study, we expressed rPcAxe2 in P. pastoris GS115, X33, and SMD1168 strains using both the native P. chrysosporium PcAxe2 fungal and α-factor secretion signals. The results showed that these recombinant P. pastoris strains did not produce rPcAxe2 in induction medium when constructed with the intrinsic secretion signal by both enzymatic assay and Western blot analysis (data not shown). The signal peptide is responsible for the translocation of the pro-protein into the endoplasmic reticulum and is subsequently cleaved by signal peptidases during the translocation process, and then the pre-protein is transported to the Golgi where the proregion is cleaved by Kex2 protease to release the mature protein (20,21). An incorrect process at this step may result in a cell that does not release rPcAxe2. Thus, the intrinsic PcAxe2 signal peptide does not have an efficient protein translocation processing in P. pastoris.

Herein, we have demonstrated that the expression levels of numerous enzymes from P. chrysosporium in P. pastoris SMD1168, a proteinase A-deficient strain, are higher than that of other P. pastoris strains such as X33 and GS115 due to reduced protein degradation (unpublished data). rPcAxe2 expression using the α-factor secretion signal was also greater in the P. pastoris SMD1168 strain. rPcAxe2 was produced after one day by 1% methanol induction. Enzyme accumulation increased dramatically by day 4 and reached 938 U l⁻¹ after 6 days of cultivation. rPcAxe2 was purified from a cell-free supernatant. The specific activity on p-nitrophenyl acetate was 39.86 U mg⁻¹, whereas the V₉₉ₐₙₐ values were 92.2 μmol and 7.4 μmol min⁻¹ ml⁻¹, respectively (Table 1). The rPcAxe2 expression level was comparable with that of rAxe from A. oryzae, but was lower than the production of rAxe from A. fuscum and Thermobifida fusca using P. pastoris as the heterologous expression host strain (10,11,13). Optimized expression conditions, including the medium, induction substrate type and concentration, pH and dissolved oxygen may result in increased production in P. pastoris.

Cellulose and xylan binding by rPcAxe2 Enzymatic assay of all fractions eluted from the column indicated there was no binding of rPcAxe2 to birchwood xylan, whereas the binding efficiency on cellulose was observed at 4.53 ± 0.35%. The main functions of CBMs are to recognize and bind polysaccharides and to increase the hydrolytic activities of the enzymes against insoluble and soluble substrates (24,25). No binding of rPcAxe2 to birchwood xylan supports the hypothesis that the catalytic module and CBM may not functionally related and evolved separately in the gene as indicated above. Gordillo et al. (26) has reported strong cellulose binding but no xylan binding for Axe from Penicillium purpurogenum containing a CBM at C-terminus.

Deglycosylation The molecular mass of purified rPcAxe2 was determined by SDS-PAGE. The results showed a single band around 63 kDa (Fig. 2). However, the theoretical molecular mass of rPcAxe2 is approximately 37 kDa. Furthermore, two potential N-glycosylation sites were found in the PcAxe2 sequence. Recombinant xylanase, mannanase, and manganese peroxidase from P. chrysosporium also express a hyperglycosylated form in P. pastoris (20,27,28). rPcAxe2 was treated with peptide-N-glycosidase and endoglycosidase H, and the results indicated that the molecular mass of rPcAxe2 was reduced to 55 kDa with the peptide-N-glycosidase (Fig. 2, lane 2) and endoglycosidase H (Fig. 2, lane 3). Thus, P. pastoris produced rPcAxe2 as a hyperglycosylated protein. The incomplete deglycosylation of rPcAxe2 may be due to O-glycosylation sites located on PcAxe2, which were found by O-β-GlcNAc attachment sites during the eukaryotic protein sequence determination using the YinOYang 1.2 tool ([http://www.cbs.dtu.dk/services/YinOYang/](http://www.cbs.dtu.dk/services/YinOYang/)). Effect of temperature and pH on rPcAxe2 activity.

Purified rPcAxe2 exhibited its highest activity at 30–35°C (Fig. 3A). The optimal temperature for rPcAxe2 was in accordance with the Axes of A. fuscum (11), Orpinomyces (8), Schizophyllum commune (29), whereas the optimal temperature of Axes from A. oryzae (13), T. fusca (10), and A. niger (30) are observed at higher values. Enzyme thermostability was examined by incubating the reaction mixture at various temperatures for 1 h. Fig. 3B shows that the enzyme did not lose activity when it was incubated at 30°C or below, but its residue activity rapidly decreased at >30°C. rPcAxe2 enzyme has an optimal pH of 7.0, which decreased rapidly when the pH was below 6.5 or above 7.5 (Fig. 3C). Numerous studies reported that a pH value of 7.0 is optimal for Axe such as A. fuscum (11), A. awamori (12), and A. niger (30), respectively. The Axes of A. oryzae (13), Orpinomyces (8), and S. commune (29) display maximum activity at pH 6.0, 9.0, and 7.7, respectively, whereas

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<th>TABLE 1. Comparison of biochemical properties of rAXE from P. chrysosporium and A. fuscum.</th>
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<td>Xylanase: specific activity (U mg⁻¹)</td>
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ND: not detectable. Esterase activity was analyzed using p-nitrophenyl acetate as substrate, while xylanase activity was examined using 1% birchwood xylan.

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the rAxe of *T. fusca* in *P. pastoris* shows its highest activity at pH 8.0 (10).

**Effect of metal ions, detergents and surfactants** Our results showed that most of the metal ions tested had an inhibitory effect on rPcAxe2 activity. rPcAxe2 activity was <50% in the presence of Mn$^{2+}$, Co$^{2+}$, Cu$^{2+}$, and Ni$^{2+}$. In particular, Fe$^{2+}$ showed strong inhibition against rPcAxe2 when the retained rPcAxe2 activity dropped to 1.6%. Ca$^{2+}$ and Mg$^{2+}$ ions had a stimulatory effect and increased activity by 9.6% and 11.9%, respectively (Fig. 4). In an Axe characterization study of *T. fusca* in the presence of 1 mM metal ions, Co$^{2+}$ and Cu$^{2+}$ did not affect enzyme activity, whereas Ca$^{2+}$ slightly decreased Axe activity (10). The effect of metal ions on Axe activity may depend on enzyme properties and metal ion concentration. For example, *S. commune* Axe is completely inhibited by 0.1–0.5 mM Ca$^{2+}$, but shows 99% relative activity at 1 mM, whereas Co$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, and Fe$^{2+}$ ions dramatically inhibit Axe by increasing concentration (29). Moreover, Mg$^{2+}$ and Ca$^{2+}$ enhance serine enzymes in which a consensus motif of the active site serine enzyme is found on PcAxe2. Triton X-100 and SDS lowered rPcAxe2 activity to 78.11% and 61.42%, respectively. However, PMSF, Tween20 and Tween80 did not have a strong effect on rPcAxe2 (retained activity >90%).
Xylanase activity

The xylan degradation ability of rPcAxe2 was examined using 1% birchwood xylan. The results indicated that xylanase specific activity of rPcAxe2 was 2 U mg\(^{-1}\) (Table 1). As Fig. 5A and B shows, the optimum pH and temperature for xylan degradation of rPcAxe2 were 5.0 and 80°C, respectively. rPcAxe2 hydrolyzed xylopentaose, releasing xylobiose and xylotriose (Fig. 5C). However, the hydrolytic activity was very low. The dual function of xylanase which has glycosyl hydrolase domain and the carbohydrate esterase domain has been reported (9). However, there is still no report on xylanase activity of Axe. Interestingly, rPcAxe2 in our study exhibited the optimal conditions for xylanase and esterase were completely different. These results suggest that PcaXe2 has two separate catalytic domains for its observed effect.

Peracetic acid biosynthesis

In situ catalyzed peracetic acid (PAA) biosynthesis by rPcAxe2 from ethyl acetate was studied. The results indicated that the synthesized PAA oxidized 58.34% of the MTS substrate. The PAA concentration catalyzed by rPcAxe2 was lower than the rAxe from A. ficuum, whereas 65.9% of MTS was oxidized by the same number of enzyme units in both reactions. However, the specific activity of rPcAxe2 was greater than that of A. ficuum rAxe (39.86 and 32.5 U mg\(^{-1}\)); thus, the ability of rPcAxe2 to catalyze PAA synthesis is higher than that of rAxe from A. ficuum when using the same amount of purified enzyme (Table 1). PAA is a selective reagent for lignin degradation. It not only converts lignin to soluble fragments by cleaving \(\beta\)-aryl ether bonds and both carbon–carbon and carbon–oxygen bonds linked to the aromatic rings but also increases water solubility of lignin by dealkylation of the O-methyl groups as well as the introduction of hydroxyl groups to the aromatic rings, and cleavage of the aromatic rings into muconic acids (31). Thus, PAA would be a great candidate for the pretreatment step during biomass degradation processing to enhance lignin removal ability.

Synergistic action with rPcXynC

In view of the applicable of rPcAxe2 on biomass degradation, we investigated the synergistic effect of rPcAxe2 and rPcXynC using xylan as substrates. As shown in Fig. 6, rPcXynC produced 338, 438 and 374 \(\mu\)g of reducing sugar when using birchwood xylan, beechwood xylan and arabinoxylan, respectively, as substrates. The combination of rPcAxe2 and rPcXynC increased the release of reducing sugar to 19.4%, 11.2%, and 6.3%, respectively. Furthermore, the pretreatment of these substrates by rPcAxe2 for 30 min enhanced the reducing sugar release by 26.4%, 17.7%, and 15.1%, respectively.

Although rPcAxe2 exhibits xylanase activity, the specific activity is very low when compared to rPcXynC (20). Moreover, the role of ester groups in the mechanism of plant cell wall resistance to enzyme hydrolysis has been clearly demonstrated earlier (32). Thus, the enhanced release of reducing sugar from xylan substrates...
by the action of rPcAxe2 can be attributed to the release of acetate
from xylan and xylooligosaccharides, rendering the rPcXynC to
rapidly hydrolyze these xylan substrates. The increased release of
sugar from biomass after supplementation of acetyl xylan esterase
into xylanase or xylanase and cellulase mixtures have been previ-
ously reported (6,7).

In this study, we have reported a novel type of acetyl xylan esterase,
rPcAxe2 which has CBM superfamily 1 from woody-
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