Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–7, 2012

www.elsevier.com/locate/jbiosc

Cloning and characterization of a novel bifunctional acetyl xylan esterase with carbohydrate binding module from *Phanerochaete chrysosporium*

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Received 13 October 2012; accepted 20 November 2012
Available online xxx

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.

[**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-xylosyl 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-xylosidase (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.l.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 ficuum* (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-L-arabinofuranosidases, and glucuronyl 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

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2012.11.018

bifunctional enzymatic characterization and synergistic action of this enzyme for xylan 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 *PcAxe2* genes constructed with expression vectors. The heterologous expression hosts *P. pastoris* X33 (wild type), GS115 (*his4*), and SMD1168 (*his4*, *pep4*) were supplied by 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. rPcAxe2 expression was carried out by inoculating recombinant *P. pastoris* in YP medium (1% yeast extract, and 2% peptone) containing 100 mM sodium phosphate (pH 6) 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 pPICZαC (Invitrogen) were used for expression in *P. pastoris*. Expression of inserts in both vectors is controlled by the methanol-inducible AOX1 promoter. pPICZα contains the signal peptide of α-secretion factor from *Saccharomyces cerevisiae*, whereas pPICZ does not contain a secretion signal.

Total cDNA synthesis Total mRNA was extracted from *P. chrysosporium* using the Oligotex mRNA Mini Kit (Qiagen, Valencia, CA, USA). Total cDNA was synthesized using the SMARTer PCR cDNA Synthesis Kit following the manufacturer's recommendations (Clontech, Mountain View, CA, USA).

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* including the signal peptide was amplified by PCR using the following forward 5′-CTCGAGATGTTCAAGCTCGCAGTC-3′ and reverse primers 5′-TACGTACGCGATGCCGAGGAAC-3′. The following conditions were employed for PCR amplification: 94°C for 1 min, 94°C for 30 s, 51°C for 30 s, and 72°C for 2 min (for 30 cycles) with *Pfu* DNA polymerase. The PCR products were A-tailed by *Taq* DNA polymerase at 72°C for 30 min and cloned to pGEM-T Easy Vector. Transformants carrying the PCR products of interest were screened by colony PCR with the specific primers mentioned above. Three recombinant plasmids were randomly isolated and sequenced. The *PcAxe2* gene was excised using *Xho*I and *Sna*BI then separated and purified on a 0.8% agarose gel. The purified *PcAxe2* gene was inserted into the pPICZC vector, resulting in pPICZC-*PcAxe2*. PCR amplification of the *PcAxe2α* cDNA into the pGEM-T Easy Vector was performed for cloning into pPICZαC using the forward primer 5′-ATCGATACAAGTGCCCGTTTGGGG-3′ and the reverse primer 5′-TCTAGATACGCGATGCCGAGGAAC-3′. The forward primer did not contain a *P. chrysosporium PcAxe2* secretion signal within the amplified region, as the *S. cerevisiae* α-secretion signal is present in pPICZαC. The PCR conditions employed were the same as previously described for *PcAxe2* cDNA. The resulting PCR product, *PcAxe2α*, was cloned into the pGEM-T Easy Vector, excised using *Cla*I and *Xba*I, purified on an agarose gel, and inserted into pPICZαC, which resulted in pPICZαC-*PcAxe2α*. After transformation into *E. coli* and isolation of the plasmid DNA, the presence of the inserts was determined both by PCR and restriction enzyme digestion followed by agarose-gel electrophoresis. A sequence analysis was performed on pPICZC-*PcAxe2* and pPICZαC-*PcAxe2α*.

To transform the yeast strain, 10 μg of plasmid was linearized using *Pme*I and transformed into *P. pastoris* 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} zeocin and 1 M sorbitol at 30°C, until colonies were observed (2–3 days). Recombinant *P. pastoris* clones were confirmed by PCR using AOX1 primers.

Expression and production of recombinant PcAxe2 (rPcAxe2) Ten *P. pastoris* transformants were cultured in 50 ml YP medium containing 1% methanol to determine extracellular enzyme activity by enzyme assay. To measure rPcAxe2 expression, a single colony was grown in 5 ml YPD medium overnight at 30°C and 200 rpm. Then, 5 ml cultures were transferred to 50 ml of fresh YP 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 YP medium. Then, the suspensions were added slowly to 90 ml of fresh YP medium supplemented with 110 mM sodium phosphate (pH 6) until an optical density of 1 was reached. Finally, fresh YP medium was added to make up the final volume to 100 ml. To induce rPcAxe2, 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 rPcAxe2, on the second day of culture, cell-free supernatants were collected by centrifugation at 2000 rpm for 5 min and filtered through 0.45 μm filters. In total, 90 ml of filtered supernatant was 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 Ni^{2+} His-tag column (Histrap, GE Healthcare, Piscataway, NJ, USA) using the ÄKTA fast protein liquid chromatography purification system. Protein was eluted with an elution buffer (20 mM sodium phosphate, 0.5 M NaCl and 500 mM imidazole; pH 7.4) and was collected as 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 rPcAxe2 rPcAxe2 (10 U) 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 shacking. The samples were applied on Poly-Prep® column (Bio-Rad, Hercules, CA, USA), and were washed with three column volumes of the buffer. In order to discard non-specific 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% cellobiose or xylobiose, respectively. Axe activity in all fractions was measured as described below.

Deglycosylation and electrophoresis The rPcAxe2 glycosylation was analyzed using endoglycosidase H and peptide-N-glycosidase F. Approximately 2 μg of purified rPcAxe2 was incubated with 500 units of enzyme for 30 min at 37°C. After the incubation, the deglycosylated rPcAxe2 was subjected to SDS-PAGE as described by Sambrook and Russell (18).

Enzymatic assay Axe 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) at 30°C. The reaction was started by adding 10 μl of 10 mmol *p*-nitrophenyl acetate and the mixture 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 Bradford method, using a Thermo Scientific Protein Assay kit (Rockford, IL, USA) with serum albumin as the standard.

Xylanase 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 acetyl xylan esterase activity.

The effect of pH on rPcAxe2 was determined by assaying for enzyme activity at 35°C with at 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 rPcAxe2 was determined by incubating the enzyme solution for 1 h at the appropriate temperatures (10–50°C).

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+}), inhibitors phenylmethanesulfonyl fluoride (PMFS) and surfactants [Tween20, Tween80, Triton X-100, and sodium dodecyl sulfate (SDS)] on rPcAxe2 activity was investigated to further characterize the enzyme. rPcAxe2 was preincubated with the aforementioned chemicals for 1 h at 35°C, and the residual activity was measured using a standard Axe assay. The final concentrations of each metal ion and PMSF used were 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 xylanase activity and xylo-oligomers hydrolysis.

The optimum temperature of 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.

Xylobiose, xylotriose or xylopentaose (Sigma, St. Louis, MO, USA) at concentrations of 5 mM, 3.3 mM or 2 mM, respectively, were incubated with 1 U of rPcAxe2 for 30 min at 80°C in 100 μl of 50 mM sodium acetate buffer at pH 5.0. The reaction was terminated by heating at 100°C for 5 min, after which 1 μl of reaction products were taken and analyzed using thin layer chromatography (TLC) on silica gel with chloroform/acetic acid/H2O (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.

Peracetic acid biosynthesis The perhydrolysis activity of rPcAxe2 was quantitatively measured by estimating the sulfoxide content using high performance liquid chromatography (HPLC) as described by Park (19). Four units of rPcAxe2 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 sulfide (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 sulfone, were analyzed by HPLC (Shimadzu, Kyoto, Japan).

Synergistic action with rPcXynC The synergistic action of rPcAxe2 on xylan (birchwood xylan, beechwood xylan and arabinoxylan) degradation was investigated using the rPcXynC (20). Xylan substrate of approximately 1 mg in 50 mM sodium acetate (pH 5.0) in 200 μl total reaction volume was used for investigation of all actions. Xylan was pretreated with 100 mU rPcAxe2 for 30 min at 37°C, followed by 200 mU rPcXynC for 10 min at 70°C, or xylan was treated simultaneously with 100 mU rPcAxe2 and 200 mU rPcXynC for 10 min at 70°C. Xylan hydrolysis using 200 mU rPcXynC for 10 min at 70°C was carried out as control. The total reducing sugar released was measured by the DNSA method as described above.

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RESULTS AND DISCUSSION

Cloning and sequence analysis of *PcAxe2* Specific primers were designed to amplify *Axe* genes from the total cDNA of *P. chrysosporium* BKM-F-1767 based on the predicted nucleotide sequences of the *P. chrysosporium* RP78 *Axe1* and *Axe2* genes. PCR products of the predicted *Axe2* gene was obtained and was named as *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 *P. chrysosporium* RP78 genome sequence. Both shared 98% nucleotide similarity. The deduced amino acid alignment showed 99% identity, whereas the cloned *PcAxe2* had four additional amino acids. The full-length cDNA nucleotide sequence of *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

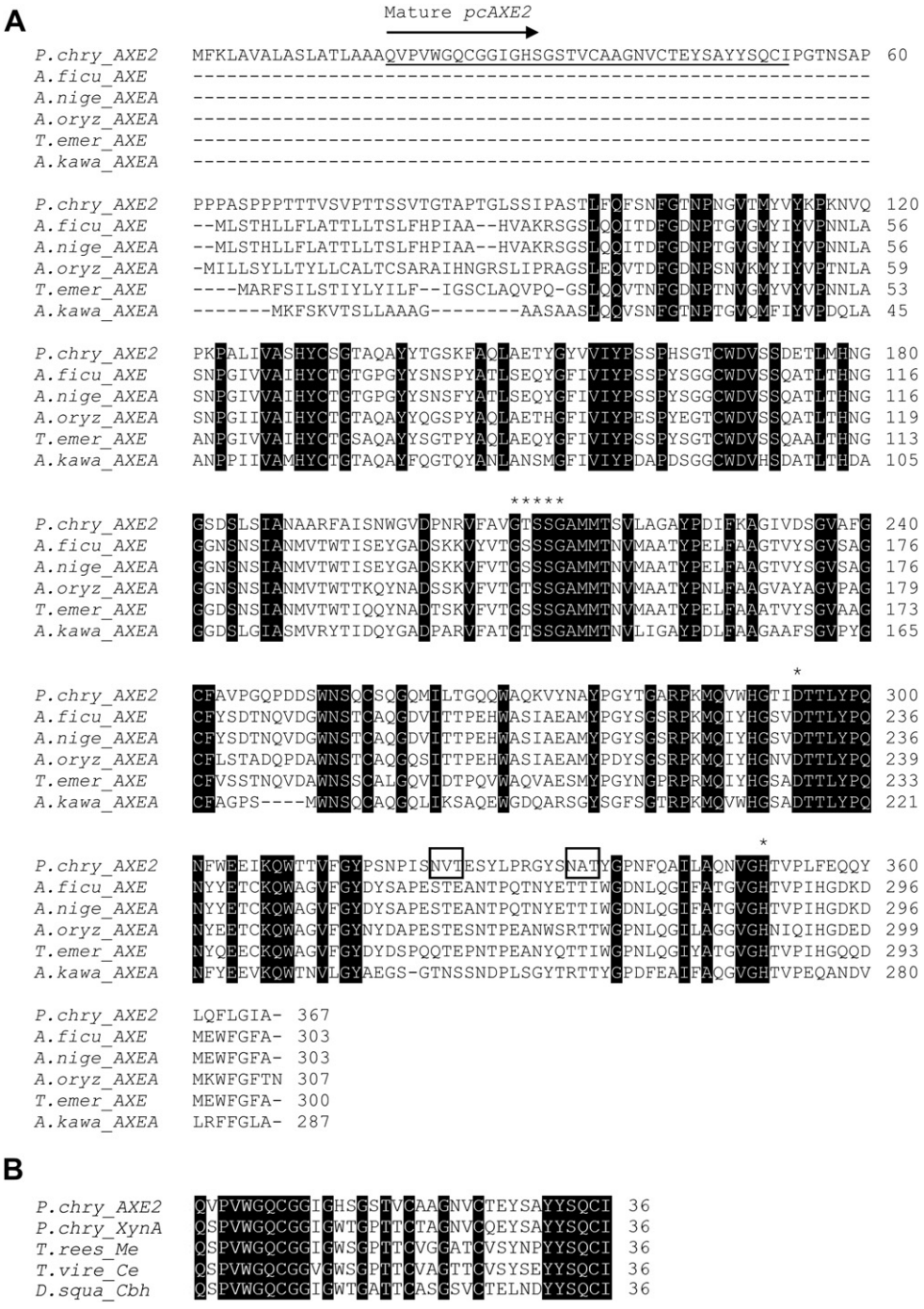

FIG. 1. A, The deduced amino acid sequence alignment of *P. chrysosporium* acetyl xylan esterase 2 (*PcAxe2*) and other *Axe*s from *A. ficuum* (AF331757), *A. niger* (XP_001395572), *A. oryzae* (XM_001826277), *Talaromyces emersonii* (HQ185193), and *A. kawachii* (GAA86514). The fungal carbohydrate 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 *PcAxe2* and others from *P. chrysosporium* endo-1,4-β-xylanase A (AAG44992), *Trichoderma reesei* carbohydrate binding module (EGR44948), *Trichoderma virens* esterase (EHK25849), and *Dichomitus squalens* cellobiohydrolaseII (EJF66131).

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serine enzyme (Gly-Thr-Ser-Ser-Gly) and two N-glycosylation sites (Asn-Val-Thr and Asn-Ala-Thr) were also found on *PcAxe2* (Fig. 1). 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/). As shown in Fig. 1, *PcAxe2* revealed a striking level of identity to *Axes* from *A. ficuum* (51%), *A. niger* (52%), *A. oryzae* (51%), *Talaromyces emersonii* (53%) and *A. kawachii* (50%). Asp294 and His351 of *PcAxe2*, which are highly conserved residues among *Axes*, are predicted to be important for catalysis (12). A fungal carbohydrate binding module (CBM) at the N-terminal region of *PcAxe2* was found by using the Scanprosite tool (http://prosite.expasy.org/scanprosite/) (Fig. 1A), whereas most Axes do not contain a CBM at its N-terminus, suggesting that *PcAxe2* may differ from other Axes. The CBM of *PcAxe2* belongs to CBM1 family based on the results of Conserved Domain Search in NCBI site (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which shows a significant identity (81–67%) with the endo-1,4-β-xylanase A (GenBank: AAG44992.1) from *P. chrysosporium*, cellobiohydrolase II (GenBank: EJF66131.1) from *Dichomitus squalens*, 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 pro-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}; the maximum rPcAxe2 activity achieved was 1058 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 K_m and V_{max} 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. ficcum* 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*.

TABLE 1. Comparison of biochemical properties of rAXE from *P. chrysosporium* and *A. ficcum*.

Factor		Species	
		P. chrysosporium	*A. ficcum*
Esterase	Optimum pH	7.0	7.0
	Optimum temperature	30–35	35–40
	Specific activity (U mg^{-1})	39.86	32.5
	K_m (μmol)	92.2	94.6
	V_{max} (μmol min^{-1} ml^{-1})	7.4	4.5
PAA biosynthesis (mM μg^{-1})		0.145	0.134
Xylanase: specific activity (U mg^{-1})		2	ND

ND: not detectable. Esterase activity was analyzed using *p*-nitrophenyl acetate as substrate, while xylanase activity was examined using 1% birchwood xylan.

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/). 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. ficcum* (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 optimum 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. ficcum* (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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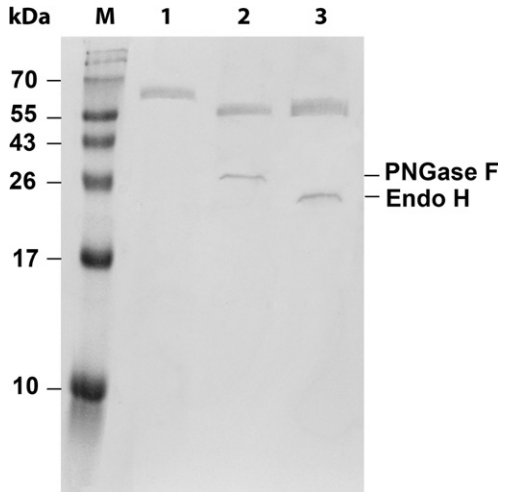

FIG. 2. SDS-PAGE analysis of purified recombinant acetyl xylan esterase 2 (rPcAxe2). Lane M, molecular mass markers; lane 1, purified rPcAxe2; lane 2, peptide-N-glycosidase F-treated rPcAxe2; lane 3, endoglycosidase H-treated rPcAxe2.

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%).

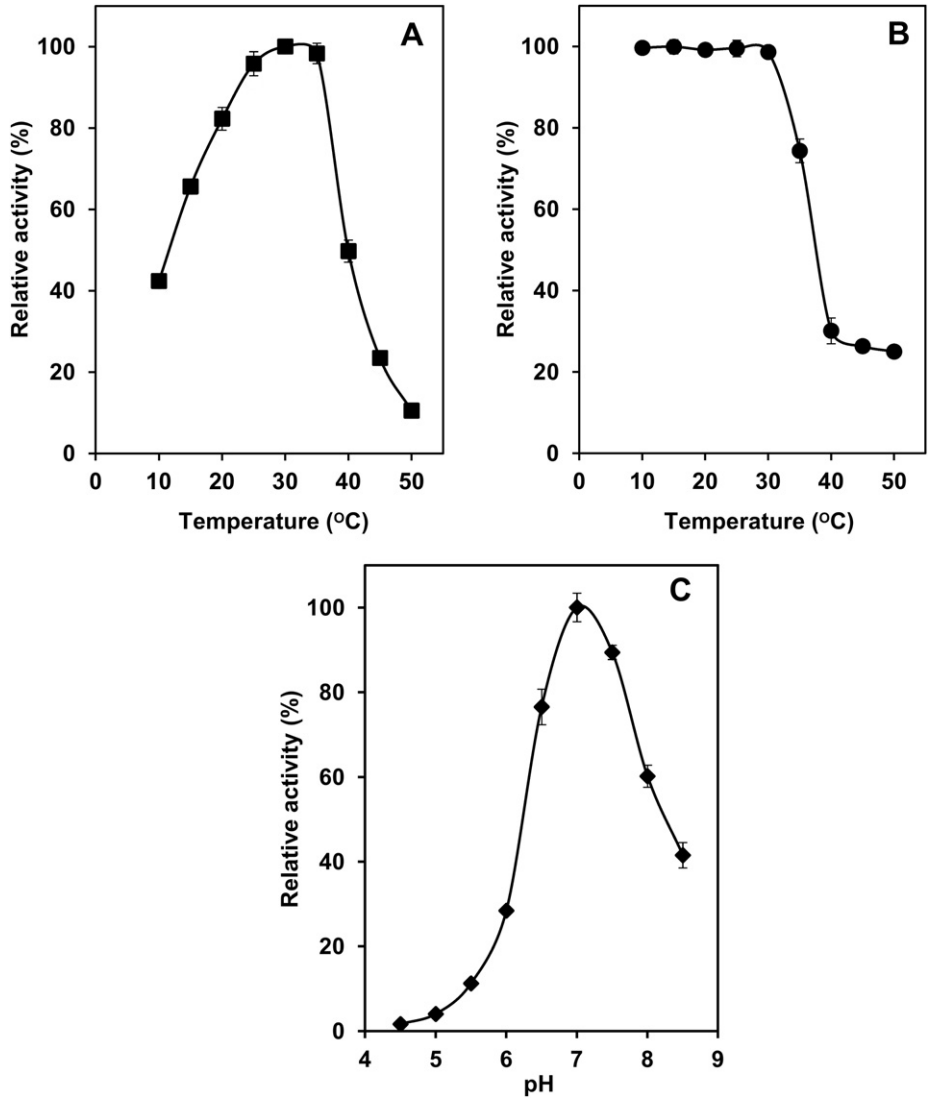

FIG. 3. Effect of temperature (A), thermostability (B), and pH (C) on esterase activity of recombinant acetyl xylan esterase 2 (rPcAxe2).

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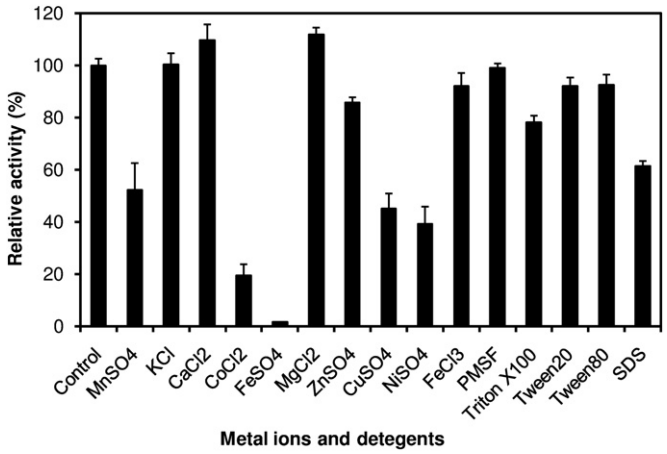

FIG. 4. Effect of metal ions on esterase activity of recombinant acetyl xylan esterase 2 (rPcAxe2). Enzyme reactions were carried out in the presence of 5 mM of each ion, 1 mM of PMSF, and 0.5% of SDS and surfactant.

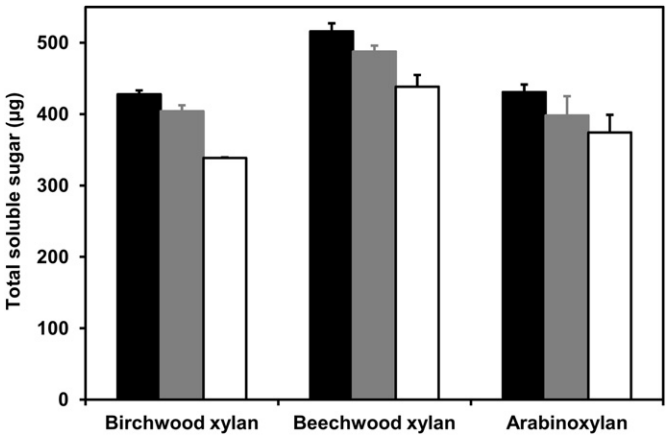

FIG. 6. Synergistic action of recombinant acetyl xylan esterase 2 (rPcAxe2) and recombinant endo-xylanase C (rPcXynC) on birchwood xylan, beechwood xylan and arabinoxylan. The black columns represent the reducing sugar released of the pretreatment by rPcAxe2 for 30 min at 37°C, following the combination with rPcXynC for 30 min, 70°C. The gray columns represent the reducing sugar released by the combined of rPcAxe2 and rPcXynC for 10 min, 70°C. The white columns represent the reducing sugar released by rPcXynC for 10 min, 70°C.

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. ficcum*, 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. ficcum* 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. ficcum* 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 β-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 µg 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 compares 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

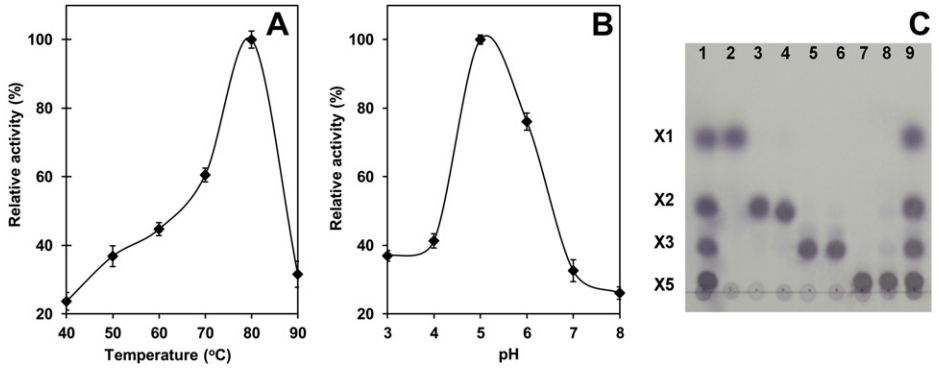

FIG. 5. Xylanase activity of recombinant acetyl xylan esterase 2 (rPcAxe2). (A) Effect of temperature; (B) effect of pH; (C) xylo-oligomers hydrolysis. The xylo-oligomers hydrolysis was carried out using xylobiose, xylotriose and xylopentaose. Lane 1 and 9, xylo-oligomers; lanes 2, 3, 5 and 7 represent xylose (X1), xylobiose (X2), xylotriose (X3) and xylopentaose (X5), respectively; lanes 4, 6, and 8 represent the xylobiose, xylotriose and xylopentaose degradation by rPcAxe2.

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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 previously reported (6,7).

In this study, we have reported a novel type of acetyl xylan esterase, rPcAxe2 which has CBM superfamily 1 from woody-degradation fungus *P. chrysosporium*. rPcAxe2 not only cleaves the acetyl group but also hydrolyzes the xylan backbone and catalyzes peracetic acid production. Furthermore, rPcAxe2 enhanced the total reducing sugar from xylan in combination with endo-xylanase. Thus, rPcAxe2 would be a useful enzyme for the degradation of lignocellulosic biomass.

ACKNOWLEDGMENTS

This research was supported by Technology Development Program for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea. We thank the Research Institute of Bioindustry at Chonbuk National University for kindly providing the facilities for this research.

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