Full Paper

Cloning and expression of a family 10 xylanase gene (*Aoxyn10*) from *Aspergillus oryzae* in *Pichia pastoris*

Xin Yin,[1] Yan-Yan Gong,[2] Jun-Qing Wang,[1] Cun-Duo Tang,[1] and Min-Chen Wu[3,*]

[1] *School of Biotechnology and Key Laboratory of Carbohydrate Chemistry & Biotechnology, Ministry of Education, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, P.R. China*
[2] *School of Pharmaceutical Science, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, P.R. China*
[3] *Wuxi Medical School, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, P.R. China*

A full-length cDNA sequence of *Aoxyn10*, a gene encoding a glycoside hydrolase (GH) family 10 xylanase from *Aspergillus oryzae*, was amplified from the total RNA by 3′ and 5′ rapid amplification of cDNA ends. The cDNA sequence is 1,689 bp, containing 5′, 3′ untranslated regions and a 1,422 bp open reading frame (ORF) that encodes a 21-aa signal peptide and a 452-aa mature peptide (designated AoXyn10). Multi-alignment revealed that AoXyn10 contains two regions: a catalytic domain (CD) and a family 1 carbohydrate-binding module (CBM1). The three-dimensional (3-D) structure of the CD was predicted by multiple template-based homology modeling. A 2,308-bp complete DNA sequence of *Aoxyn10* was obtained from the genomic DNA by both pUCm-T vector-mediated and conventional PCRs, harboring 5′, 3′ flanking regulatory regions, five exons and four introns. Moreover, *Aoxyn10* was extracellularly expressed in *Pichia pastoris*. One transformant labeled as *P. pastoris* GS/Xyn4-11 was selected, expressing the highest recombinant AoXyn10 (named reAoXyn10) activity of 45.0 U/ml. SDS-PAGE analysis revealed that reAoXyn10, a glycoprotein with an apparent molecular weight (M.W.) of about 56.0 kDa, was secreted into the cultured medium. The purified reAoXyn10 displayed the maximum activity at pH 5.5 and 60°C. It was stable at a pH range of 4.0−7.0, and at 50°C or below. Its activity was not affected by an array of metal ions or EDTA, but was inhibited by Mn^{2+} and Ba^{2+}. The K_m and V_{max} of reAoXyn10 were 1.7 mg/ml and 817 μmol/min/mg, respectively.

Key Words——*Aspergillus oryzae*; cloning; expression; *Pichia pastoris*; pUCm-T vector-mediated PCR; xylanase

Introduction

Xylans belong to a highly complex and variable polysaccharide family, containing a backbone of β-1,4-

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* Corresponding author: Dr. Min-Chen Wu, Wuxi Medical School, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, P.R. China.
 Tel: +86−510−85329042   Fax: +86−510−85329042
 E-mail: biowmc@126.com
 Xin Yin and Yan-Yan Gong, the two first authors, contributed equally to this work.

D-linked xylopyranosyl residues partially replaced by acetyl, 4-*O*-methyl-D-glucuronosyl and L-arabinofuranosyl groups. Owing to their heterogeneity and complexity, the complete degradation of xylans requires the synergistic action of several xylanolytic enzymes, such as endo-β-1,4-D-xylanase (EC 3.2.1.8), β-D-xylosidase (EC 3.2.1.37) and α-L-arabinofuranosidase (EC 3.2.1.55). Among them, endo-β-xylanase, generally abbreviated to xylanase, is a key enzyme in that it can cleave the internal β-1,4-D-xylosidic linkages of xylan backbones (Khandeparker and Numan, 2008). To date, various xylanases have been applied in the

food, paper, feed, brewing and textile industries, and in the production of xylooligosaccharides and bioconversion of lignocellulose into biofuel (Fawzi, 2010).

Numerous xylanases have been isolated and characterized from microorganisms, plants and rumen microbiota, among which filamentous fungi were recognized to have a great potential for the industrial production of xylanases (Polizeli et al., 2005). Some xylanase genes from filamentous fungi, such as *Aspergillus niger* (Ruanglek et al., 2007), *A. terreus* (Chantasingh et al., 2006), *Penicillium thiersii* (Cui et al., 2009) and *Fusarium oxysporum* (Moukouli et al., 2011), have been cloned and expressed in heterologous cells. Based on the structure alignment and hydrophobic cluster analysis, xylanases have been mainly classified into GH families 10 and 11 (Collins et al., 2005), while enzymes with xylanolytic activity were also widespread in GH families 5, 7, 8, 16, 26, 30, 43, 52 and 62 (http://www.cazy.org/fam/acc_GH.html) (Luo et al., 2010). Compared to GH family 11 counterparts, the family 10 xylanases exhibited higher molecular weight ($>$ 30 kDa) and lower substrate specificity, and also had catalytic activities towards some cellulosic substrates, such as aryl cellobioside (Biely et al., 1997). The overall 3-D structure of GH family 10 xylanases was similar to that of family 5 enzymes, consisting principally of the $(\beta/\alpha)_8$ barrel fold (Larson et al., 2003).

In our previous studies, the gene encoding GH family 10 xylanase A from *Aspergillus usamii* E001, *Auxyn10A*, was cloned, analyzed and expressed in *P. pastoris* GS115 (Wang et al., 2013). In this work, we report the cloning and analysis of both the full-length cDNA and complete DNA sequences of *Aoxyn10* from *A. oryzae* CICC40186. Simultaneously, the 3-D structure of the AoXyn10's CD is modeled using the SALIGN and MODELLER 9.9 programs. Moreover, extracellular expression of the *Aoxyn10* in *P. pastoris* GS115 and enzymatic properties of the purified reAoXyn10 are described.

Materials and Methods

Strains, vectors and media.   *Aspergillus oryzae* obtained from the Center of Industrial Culture Collection of China (Accession No. CICC40186) was cultured in a medium (1% tryptone, 0.5% yeast extract, 1% dextrose and 0.5% birchwood xylan, pH 6.0) for total RNA and genomic DNA extraction. *E. coli* JM109 and pUCm-T vector (Sangon, Shanghai, China) were used for gene cloning, and *E. coli* DH5α and pPIC9K^M vector (Wang et al., 2013) for construction of the recombinant expression vector. LB medium (1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.2) was used to grow *E. coli* JM109 and DH5α. *Pichia pastoris* and its transformants were cultured and induced in the following media prepared according to the manual of the Multi-Copy Pichia Expression Kit (Invitrogen, San Diego, CA): yeast extract peptone dextrose (YPD), minimal dextrose (MD), geneticin G418-containing YPD, buffered glycerol-complex (BMGY) and buffered methanol-complex (BMMY).

Total RNA and genomic DNA extraction.   After *A. oryzae* CICC40186 was cultured at 30°C for 36 h on a rotary incubator (220 rpm), the mycelia were collected through filtration, and thoroughly washed with sterile deionized water. The total RNA was extracted from mycelia by using the RNA Extraction Kit (Sangon). Extraction of the *A. oryzae* genomic DNA was performed according to the method reported previously (Wu et al., 2011).

Primers for PCR amplification.   After aligning four family 10 xylanase sequences from fungi of *A. fumigatus* Af239, *Neosartorya fischeri* NRRL181, *A. terreus* NIH2624 and *A. clavatus* NRRL1, the two most conserved segments at the N- and C-terminal regions, KLYYNDY and TIWDWTDKYSWVPS, were located. A pair of degenerate primers X10F1 and X10R1 was designed corresponding to KLYYNDY and WDWTDKY (Table 1). Primers dT-MP and MP (original names, Oligo dT-M13 Primer M4 and M13 Primer M4) as well as primers OP and IP (original names, 5′ RACE Outer Primer and 5′ RACE Inner Primer) were provided by the RNA PCR Kit and 5′-Full RACE Kit (TaKaRa, Dalian, China), respectively. A pair of specific primers X10F and X10R with *Xho*I and *Not*I sites, respectively, was used to amplify the AoXyn10-encoding gene. Using the pUCm-T vector-mediated PCR technique, a 5′ flanking DNA sequence of *Aoxyn10* was amplified with primers Tv-5frsF (identical to the 21-bp fragment upstream the T/A cloning site in pUCm-T vector), X10R1 and X10R2, while a 3′ flanking DNA sequence was cloned with X10F2, X10R3 and Tv-3frsR (complementary to the 20-bp fragment downstream the T/A cloning site). As listed in Table 1, all PCR primers (except those provided by Kits) were synthesized by Sangon (China).

Cloning of the full-length cDNA.   Firstly, a 3′-end cDNA fragment of *Aoxyn10* was amplified by using the

Table 1. Sequences of the primers for PCR amplification.

Fragments	Primers	Sequences (5′-3′)[a]	Size (bp)
3′-end cDNA	X10F1	ARCTVTACTACAACGACTAC	20
	X10R1	TACTTRTCRGTCCAGTCCC	19
	dT-MP	GTTTTCCCAGTCACGAC-Oligo dT	37
	MP	GTTTTCCCAGTCACGAC	17
5′-end cDNA	OP	CATGGCTACATGCTGACAGCCTA	23
	IP	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	34
	X10R1	TACTTRTCRGTCCAGTCCC	19
	X10R2	GCCGTAGGACTGGATTAGC	19
Central DNA	X10F1	ARCTVTACTACAACGACTAC	20
	X10R3	TTAGGCTTGGATACACTGC	19
5′flanking DNA	Tv-5frsF	ACGACTCACTATAGGGCGACA	21
	X10R1	TACTTRTCRGTCCAGTCCC	19
	X10R2	GCCGTAGGACTGGATTAGC	19
3′flanking DNA	X10F2	TTCTTCCAGCTCTCCCAG	18
	X10R3	TTAGGCTTGGATACACTGC	19
	Tv-3frsR	CTATGACCATGATTACGCCA	20
AoXyn10-encoding gene	X10F	<u>CTCGAG</u>AAAAGAGCTGGTCTGCATGACGC	29
	X10R	<u>GCGGCCGC</u>TTAGGCTTGGATACACT	25

[a]R=A/G, V=G/A/C.

RNA PCR Kit (Wu et al., 2011). In brief, the first-strand cDNA from the total RNA was reversely transcribed with primer dT-MP. Using the resulting first-strand cDNA as the template, the first-round PCR was carried out with X10F1 and MP under following conditions: a denaturation at 94°C for 2 min; 30 cycles of at 94°C for 30 s, 52°C for 30 s, and 72°C for 75 s; and an elongation at 72°C for 10 min. Each amplified band was agarose gel-purified, and subjected to the second-round PCR with X10F1 and X10R1 for confirmation (nested PCR). The amplified target band was inserted into pUCm-T vector, and transformed into *E. coli* JM109, followed by DNA sequencing. Next, a 5′-end cDNA fragment was amplified by using the 5′-Full RACE Kit according to the manufacturer's instructions. The first-strand cDNA was used as the template for the first-round PCR with OP and X10R1, and subjected to the second-round PCR with IP and X10R2 for confirmation. Finally, a full-length cDNA sequence of *Aoxyn10* was obtained by assembling the amplified 5′- and 3′-end cDNA fragments.

Cloning of the complete DNA. Firstly, 5′ and 3′ flanking DNA sequences of *Aoxyn10* were amplified by pUCm-T vector-mediated PCR as follows. The *A. oryzae* genomic DNA was separately digested with *Msp*I (CCGG) and *Ava*II (GGA(/T)CC), which were selected by pre-experiments, for the cloning of 5′ and 3′flank-

ing sequences. The cohesive ends of digested DNA fragments were filled in and an adenine nucleotide (A) at 3′-ends was added with *Ex Taq* DNA polymerase at 72°C for 10 min, followed by their insertion into pUCm-T vector. Using recombinant pUCm-T vectors as templates, a 5′flanking sequence was amplified by the first-round PCR with Tv-5frsF and X10R1, and subjected to the second-round PCR with Tv-5frsF and X10R2 for confirmation. Similarly, a 3′flanking sequence was amplified by the first-round PCR with X10F2 and Tv-5frsR, and confirmed by the second-round PCR with X10F2 and X10R3. Next, a central sequence was directly amplified from the genomic DNA by conventional PCR with X10F1 and X10R3. All amplified target bands were agarose gel-purified, and inserted into pUCm-T, followed by DNA sequencing. Finally, a complete DNA sequence of *Aoxyn10* was obtained by assembling the three cloned DNA fragments.

Analysis of the DNA and amino acid sequences. The localization of exon/intron boundaries was predicted using the GeneMark program (http://opal.biology. gatech.edu/GeneMark/eukhmm.cgi). The prediction of the 5′ promoter region and its characterization were carried out using the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html), together with the PLACE program (http://www.dna.affrc.go.jp/PLACE/signalscan.html). The ORF was determined

using the NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). A signal peptide sequence was predicted using SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/). Putative N-linked glycosylation sites were located using the NetNGlyc 1.0 program (http://www.cbs.dtu.dk/services/NetNGlyc/). Physicochemical properties were identified using the Protparam program (http://au.expasy.org/tools/protparam.html). Homology sequence searches in the NCBI website (http://www.ncbi.nlm.nih.gov/) were performed using the BLAST server. Multi-alignment among family 10 xylanase primary structures was carried out using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and DNAMAN 6.0. Eighteen known sequences of GH family 10 xylanases were randomly selected from the Swiss-Prot Protein Knowledgebase (http://www.ebi.ac.uk/swissprot/). The phylogenetic tree was constructed using the ClustalW2 program and MEGA 4.0 software. Putative disulfide bridges were located using the DiANNA 1.1 program (http://clavius.bc.edu/~clotelab/DiANNA/).

Multiple template-based homology modeling. Based on the results of homology searches among family 10 xylanases in the Protein Data Bank (PDB), three known crystal structures of xylanases, having high sequence identities with AoXyn10, from *Cellulomonas fimi* (3CUF), *Streptomyces olivaceoviridis* E-86 (1V6Y) and *Thermotoga maritima* (1VBR) were selected as templates. The 3-D structure of AoXyn10's CD was predicted by multiple template-based homology modeling using both the SALIGN program (http://salilab.org/DBAli/?page=tools_&action=f_salign) and MODELLER 9.9 program (http://salilab.org/modeller/). The quality of the 3-D model was assessed using the PROCHECK program (http://nihserver.mbi.ucla.edu/SAVES/PROCHECK).

Nucleotide sequence accession number. A complete DNA sequence of *Aoxyn10* along with the deduced amino acid sequence of AoXyn10 has been deposited in the GenBank database under accession number JX101334.

Construction and transformation of the recombinant expression vector. Based on the analytical results of the cloned full-length cDNA, X10F and X10R were designed and used to amplify the AoXyn10-encoding gene under the following conditions: a denaturation at 94°C for 2 min; 30 cycles of at 94°C for 30 s, 54°C for 30 s, and 72°C for 90 s; and an elongation at 72°C for 10 min. The target PCR product was digested with

*Xho*I and *Not*I, and inserted into pPIC9K^M vector digested with the same enzymes, followed by its transformation into *E. coli* DH5α. The recombinant expression vector, named pPIC9K^M-*Aoxyn10*, was confirmed by DNA sequencing. Mediated by pPIC9K^M vector (Wang et al., 2013), the expressed reAoXyn10 could retain a native N-terminus of AoXyn10. The resulting pPIC9K^M-*Aoxyn10* was linearized with *Sal*I, and transformed into *P. pastoris* GS115 by electroporation on a Gene Pulser apparatus (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. *P. pastoris* GS115 transformed with pPIC9K^M vector was used as the negative control (labeled as *P. pastoris* GSXC).

Screening and expression of the P. pastoris transformants. All *P. pastoris* transformants were primarily screened based on their ability to grow on a MD plate, and then successively inoculated on G418-containing YPD plates at increasing concentrations for screening multiple copies of integration of *Aoxyn10* into the *P. pastoris* genome. Extracellular expression of *Aoxyn10* in *P. pastoris* GS115 by an α-factor secretion signal in pPIC9K^M was performed according to the instructions of the Multi-Copy Pichia Expression Kit (Invitrogen) with slight modification (Li et al., 2011). In brief, each single colony of *P. pastoris* transformants was inoculated into 25 ml of BMGY medium, and cultured at 30°C with 220 rpm until the OD$_{600}$ reached 2−4. Then, the yeast cells were harvested, resuspended in 30 ml of BMMY medium, and induced for 96 h at 30°C for the reAoXyn10 expression by adding methanol to a final concentration of 2.0% (v/v) at 24 h intervals. Among all transformants tested, one strain expressing the highest reAoXyn10 activity, labeled as *P. pastoris* GS/Xyn4-11, was selected.

Enzyme activity and protein assays. Xylanase activity was assayed by measuring the amount of reducing sugars released from birchwood xylan (Sigma, St. Louis, MO), using the 3,5-dinitrosalicylic acid (DNS) method as described previously (Wu et al., 2005). One unit (U) of xylanase activity was defined as the amount of enzyme liberating 1 μmol of reducing sugar equivalent per min under the standard assay conditions (at pH 4.6 and 50°C for 10 min). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Laemmli (1970) on a 12.5% gel, and the isolated proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma). The protein concentration was measured with the BCA-200 Protein Assay Kit (Pierce, Rockford, IL), us-

ing bovine serum albumin as the standard.

Purification of the expressed reAoXyn10. After GS/Xyn4-11 was induced by methanol for 96 h, a total of 40 ml of cultured supernatant was brought to 75% saturation by adding solid ammonium sulfate, followed by centrifugation. The precipitate was harvested, dissolved in 4.0 ml of 20 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 6.0), and dialyzed against the same buffer overnight. The dialysate was concentrated to 1.0 ml by ultrafiltration using a 10-kDa cut-off membrane (Millipore, Billerica, MA), and loaded onto a Sephadex G-75 column (Amersham Pharmacia Biotech, Uppsala, Sweden; 1.6×80 cm), followed by elution with the same buffer (pH 6.0) at a flow rate of 0.4 ml/min. Aliquots of 2.0 ml eluent only containing reAoXyn10 were pooled and concentrated. All purification procedures were performed at 4°C unless otherwise stated.

Carbohydrate content and N-deglycosylation assays. The carbohydrate content of the purified reAoXyn10 expressed by *P. pastoris* GS/Xyn4-11 was assayed by the phenol-sulfuric acid method (Dubois et al., 1956), using D-mannose as the standard. Endoglycosidase H (New England Biolabs, Ipswich, MA) can catalyze the release of N-linked oligosaccharides, by which N-deglycosylation of the glycoprotein was performed according to the manufacturer's instructions. The purified reAoXyn10 was treated at 100°C for 10 min, cooled and incubated with endoglycosidase H at 37°C for 1 h.

Enzymatic properties of the purified reAoXyn10. The pH optimum of purified reAoXyn10 was measured under standard assay conditions, except for 0.5% of birchwood xylan in 50 mM Na_2HPO_4-citric acid buffer at a pH range of 3.0−7.5. To estimate the pH stability, aliquots of reAoXyn10 were incubated at 40°C for 1 h at varied pH values (3.0−7.5). Its pH stability in this work is defined as a pH range, over which the residual activity of reAoXyn10 is more than 85% of its original activity.

The temperature optimum of purified reAoXyn10 was assayed, at pH optimum, at temperatures ranging from 45 to 70°C. Aliquots of reAoXyn10 were incubated in the absence of substrate for 30 min at different temperatures (40−65°C). Here, its thermostability is defined as a temperature at or below which the residual activity of reAoXyn10 retains over 85% of its original activity.

To evaluate the effects of metal ions and EDTA on its activity, aliquots of purified reAoXyn10 were incubated at 40°C with various metal ions and EDTA, respectively, at a final concentration of 5.0 mM in 20 mM Na_2HPO_4-citric acid buffer (pH 5.0) for 1 h. The residual activity of reAoXyn10 was assayed under standard conditions. The enzyme solution without any additive was used as the control.

The hydrolytic reaction rate (μmol/min/mg) of purified reAoXyn10 was determined under standard assay conditions, except for the concentrations of birchwood xylan ranging from 1.0 to 10 mg/ml. The hydrolytic rate versus substrate concentration was plotted to verify whether the hydrolytic mode of reAoXyn10 conformed to the Michaelis-Menten equation. The kinetic parameters, K_m and V_{max}, were graphically determined from the Lineweaver-Burk plotting.

Results and Discussion

Analysis of the full-length cDNA and complete DNA

The full-length cDNA sequence of *Aoxyn10* is 1,689 bp in length (except polyA), containing a 104 bp 5′ untranslated region, a 1,422 bp ORF, and a 163 bp 3′ untranslated region in which a putative polyadenylation signal (AATAAA) is located at 144 bp downstream the stop codon (TAA). Besides, there exist four introns ranging from 57 to 75 bp in the DNA sequence (Fig. 1). All exon/intron boundaries except one conform to the canonical GT-AG rule. There was predicted, designating the starting point of transcription (A) as +1, to be a 5′ promoter region of *Aoxyn10* located over a range from −40 to +10. A TATAAG sequence in the promoter that functions as a substitute for the classical TATA box (TATAAA) is located −30 bp upstream the starting point, which was in agreement with the consensus distance generally found in eukaryote promoters. Other consensus sequences, such as TTATTT, can also act as substitutes for the classical TATA box (Lampidonis et al., 2008). In eukaryotes, CAAT boxes are typically found about −75 bp upstream the starting point, while some may be located further from the starting point (Wenkel et al., 2006). In some cases, such as the *Auman5A* encoding a β-mannanase (AuMan5A) from *A. usamii* YL-01-78, there was no CAAT box (Tang et al., 2011). Coincidentally, in this work, no CAAT box was found in the *Aoxyn10* until −166.

Analysis of the AoXyn10 primary structure

SignalP 4.0 predicted an unambiguous signal peptide cleavage site between Leu[21] and Ala[22]. Together

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-166   CCGGCATTAATTCTCCGCGATGGAGTCTTGCGATGCCGATTAAATGAAACTGAGGCTAGGGCAGAGGCCATCCAT
-91    GGTTTAATTTAGCTTGCTTCAGCTTATCTGCGAAGGTCGACTTAGTCTAATTGGGAATTCGTATAAGTTGTTTGG
-16    AGTCGACCTCGACATAAGCATTCCTCCTGTTAACTCTTTTCACTCCTTAAGCTGTGATCCTGGTGATCAGATCGT
60     CCAACTCTTACCTTCTTTTCTTCTTTTTTGTCAAGTAATAATTGCAATGCGTTTCCAGCCTTTGACCACGGCTCTG
                                                          M   R   F   Q   P   L   T   T   A   L
135    GCTGTGGGTGTCTCTGCCCATCTGTTCGTTcccctgttatttctaacttaatatccgatcttaagccgagtacag
       A   V   G   V   S   A   H   L   F   V
210    atactaacgaagaattgaagtgctcaagcaCTTGCTGGTCTGCATGACGCTGCCGTCGCAAAGGGACTGCTCTAC
                                         ──────────────────────────▶
                                                X10F
                                      L   A   G   L   H   D   A   A   V   A   K   G   L   L   Y
285    TTCGGTACCGCAACCGATAACCCCGAGCTCACCAACACCTCCTATGTCACCCAGCTGAACAACACGGGTGACTTT
          F   G   T   A   T   D   N   P   E   L   T   N   T   S   Y   V   T   Q   L   N   N   T   G   D   F
360    GGTCAGATCACTCCTGGAAACTCGCAGAAGgtgaggtctagttgccatcctgaaggatttaaagaatatgaatgg
          G   Q   I   T   P   G   N   S   Q   K
435    ctgatgcagttgaatgaaaccaatagTGGGACTCAACCGAGCCGTCTCAGAATGAATTCTCCTTCACCAACGGCG
                                      W   D   S   T   E   P   S   Q   N   E   F   S   F   T   N   G
510    ACGTCATTGCCGATCTGGCCGACGCCAATGATCAGAAGCTGAGATGCCATAACTTGGTCTGGCACCAGCAGCTGC
          D   V   I   A   D   L   A   D   A   N   D   Q   K   L   R   C   H   N   L   V   W   H   Q   Q   L
585    CTAACTGGgtatgctctatttttcgcgtgcctccgtgatggtagatatcggatgctaatcaggacagGTTTCGAG
          P   N   W                                                              V   S   S
660    CGGGTCCTGGACCAACGAAACCCTAACTGCCGTTCTGCAGAACCATATCACAAATGTTGTGAAGCACTACAAGGG
          G   S   W   T   N   E   T   L   T   A   V   L   Q   N   H   I   T   N   V   V   K   H   Y   K   G
735    ACGATGTTACGCATGGGATGTAGTGAACGAGgtactttgttatctcctagcatgcccattagagcacagatat
          R   C   Y   A   W   D   V   V   N   E
810    tgactcgaaacagGCCCTGGCCGACGACGGCTCCTACCGCGACTCCATCTGGTACAAAACCGTCGGCGAGGCGTA
                         A   L   A   D   D   G   S   Y   R   D   S   I   W   Y   K   T   V   G   E   A   Y
885    CATTCCCATCGCCTTCGCAGCTGCCGCTGCCGCCGACAAAGACGTGAAGCTGTACTACAACGACTACAGCATCGA
                                                                  ──────────────────────────▶
                                                                          X10F1
          I   P   I   A   F   A   A   A   A   A   A   D   K   D   V   K   L   Y   Y   N   D   Y   S   I   E
960    GTGGGGCGGTTCCAAGTCCACCGCCGCCCAGAACATCGTCAAGCTAATCCAGTCCTACGGCGGTAAGATCGACGG
                                                   ◀──────────────────────────
                                                           X10R2
          W   G   G   S   K   S   T   A   A   Q   N   I   V   K   L   I   Q   S   Y   G   G   K   I   D   G
1035   TGTCGGTCTACAGGCCCATTTCACCGTCGGCCAAACCCCCGCCCGCAAGGACCTCGCCAGCAACCTGAAGGCCTT
          V   G   L   Q   A   H   F   T   V   G   Q   T   P   A   R   K   D   L   A   S   N   L   K   A   F
1110   CACCGACCTCGGCGTAGAGGTCGCCTACACCGAGGTTGACGTGCGTATGGAGACCCCAGCGACGGACGCCAACCT
          T   D   L   G   V   E   V   A   Y   T   E   V   D   V   R   M   E   T   P   A   T   D   A   N   L
1185   GCAGCAGCAGAGCACCGAATTCGCCAACATCGTCGGTGCTTGTCTCGATACCGAGGGCTGTGTCGGTGTTACCAT
          Q   Q   Q   S   T   E   F   A   N   I   V   G   A   C   L   D   T   E   G   C   V   G   V   T   I
1260   TTGGGACTGGACCGACAAGTACTCCTGGGTGCCCAGCACATTCCCAGGCTACGGCGCTGCCTGCCCCTGGGACGA
          ◀──────────────────────────
            X10R1
          W   D   W   T   D   K   Y   S   W   V   P   S   T   F   P   G   Y   G   A   A   C   P   W   D   E
1335   GAACTTTGAGAAGAAGCCCGCTTACCAGGGCATTTTGGAGGTTTTGGGAGGTGAGGCTTCTGCTTCTACTTCTAC
          N   F   E   K   K   P   A   Y   Q   G   I   L   E   V   L   G   G   E   A   S   A   S   T   S   T
1410   TTCTGTGTCTGCTTCTGCTTCTGCACCTGAGACCACTTCCGCCATTATCGCTACCACTGCTGCCGCTGTCACCTC
          S   V   S   A   S   A   S   A   P   E   T   T   S   A   I   I   A   T   T   A   A   A   V   T   S
1485   TACTTCTTCTTCCAGCATCAGCACCAGCACAAGCGTCAGCTCCAGCATTCCTGCCGCTCCTACTTCTTCCAGCTC
                                                                     ──────────────────────────
                                                                             X10F2
          T   S   S   S   S   I   S   T   S   T   S   V   S   S   S   I   P   A   A   P   T   S   S   S   S
1560   TCCCAGCACTACCCTGGCCACCTCATCCAGAACTTCCTCCGCCATTCCTTCCTCCTCTTCTTCAGCCACGCCAAC
       ─────
          P   S   T   T   L   A   T   S   S   R   T   S   S   A   I   P   S   S   S   S   A   T   P   T
1635   TGACTTCATCCCTCAGCCTAGTTCAACCGCCACCGGCCGCGTCAAGGTCTACTACCAGTGCGGCGGTATTAACTA
          D   F   I   P   Q   P   S   S   T   A   T   G   R   V   K   V   Y   Y   Q   C   G   G   I   N   Y
1710   CCAGGGTAGCACGGAGTGCGAGGAGGGTCTGACCTGCAAGAAGTGGAACCCTTACTATTCGCAGTGTATCCAAGC
                                                                  ◀──────────────────────────
                                                                         X10R3/X10R
          Q   G   S   T   E   C   E   E   G   L   T   C   K   K   W   N   P   Y   Y   S   Q   C   I   Q   A
1785   CTAAGCTTATTTATATACGCTTTATGAATGGTTCGTTGAATCACGAGTGTTTATCAGTGGTTTTGGCTTAGATGT
       *
1860   TTTATCTTGCGCTCTGGATTATATAGTTACTATTACTATAGTCTTGAGTTCAAGATAAAACATATATAAAATAAA
1935   TCTTTTCCTTTGATAAACAGAGACAATAGAGATACCTGCTGTCGGTAGGAAGCTATAATAGGGAAATAGCACACAA
2010   ATAGAACAAACCCCGATAACAAAACTCAAACTCCCAACCCAATAACCTCCTCCCCCCTCCAGACCCTAATAAAAG
2085   TAAACTCACATAAACAATCCCTCCACCACGAACTCAAACCCCAGCAAACCCCAGGTCC
```

Fig. 1.   Both the full-length cDNA and complete DNA sequences of *Aoxyn10* from *A. oryzae* CICC40186, and the AoXyn10 amino acid sequence deduced from *Aoxyn10*.

Four introns ranging from 57 to 75 bp are shown in lowercase letters. A signal peptide from Met[1] to Leu[21] is underlined. The bold letters TATAAG and A in boxes indicate the putative TATA box and starting point of transcription. The gray italic letters ATG and TAA represent the start and stop codons, respectively. A putative polyadenylation signal, AATAAA, is shown as gray underlined letters. The CCGG and GGA(/T)CC in boxes indicate restriction enzymes *Msp*I and *Ava*II sites, respectively. The bold arrows below the letters indicate various primers for PCR amplification.

with the multi-alignment result of the N-terminal regions of family 10 xylanases, it was concluded that 473-aa preAoXyn10 consists of a 21-aa signal peptide and a 452-aa AoXyn10 that contains two regions: a 317-aa CD and a 38-aa CBM1 (Levasseur et al., 2005). At the C-terminal region of AoXyn10, there is a very long (97 aa) Ser/Thr-rich linker between the CD and CBM1 (Fig. 2). The theoretical M.W. of 48,108 Da and
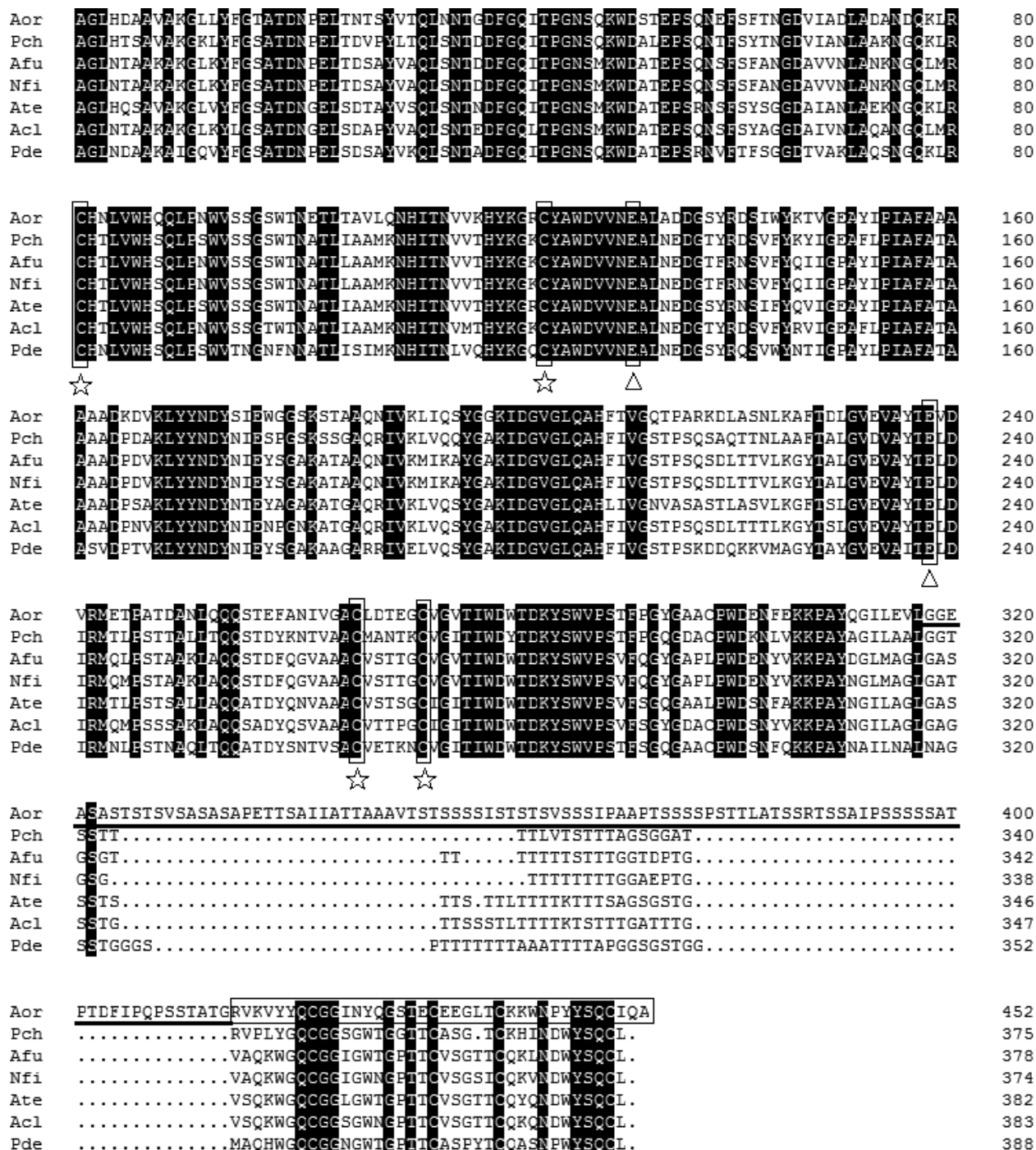

Fig. 2. Multi-alignment of AoXyn10 with six other fungal family 10 xylanases.

Aor, *A. oryzae* CICC40186 (in this work); Pch, *P. chrysogenum* Wisconsin 54-1255; Afu, *A. fumigatus* Af293; Nfi, *N. fischeri* NRRL181; Ate, *A. terreus* NIH2624; Acl, *A. clavatus* NRRL1 and Pde, *P. decumbens*. Identical amino acids are indicated by solid black boxes. The sequence of a linker is underlined and a CBM1 is shown in a box. Two catalytic residues, Glu[131] and Glu[238], are indicated by triangles. Two disulfide bridges, Cys[81]-Cys[123] and Cys[266]-Cys[272], are indicated by stars.

pI of 4.63 were calculated from the deduced AoXyn10 sequence. There are three putative N-glycosylation sites in AoXyn10 at positions N^{26}-T^{27}-S^{28}, N^{34}-N^{35}-T^{36} and N^{101}-E^{102}-T^{103}. Sequence alignment revealed that identities of AoXyn10 with GH family 10 xylanases of *P. chrysogenum* Wisconsin 54-1255 (XP_002564127), *A. fumigatus* Af239 (XP_751237), *A. clavatus* NRRL1 (XP_001268415) and *P. decumbens* (ADX86896) are 71.9, 70.4, 67.5 and 65.6%, respectively (Fig. 2). Furthermore, four conserved motifs in all fungal family 10 xylanases, RGHTLVWHSQLP, YAWDVVNE, KLYINDY and IDGIGSQTH (Chantasingh et al., 2006), were also recognized in the AoXyn10 sequence. These features implied that AoXyn10 is a member of family 10. Based on the topology of the phylogenetic tree, AoXyn10 is most closely relative to that of *P. chrysogenum* Wisconsin 54-1255 but very far from those of bacteria (Fig. 3).

Modeling and analysis of the AoXyn10 3-D structure

Amino acid sequence identities of AoXyn10 with known crystal structure xylanases from *C. fimi*, *S. olivaceoviridis* E-86 and *T. maritima* were more than 50%, indicating that they are suitable as templates for homology modeling of AoXyn10. Studies verified that the validity of multiple template-based homology modeling relied mainly on both the primary structure identity between the target and template proteins and accuracy of multi-alignment of template crystal structures (Madhusudhan et al., 2009). In contrast to the single template-based modeling method, the multiple template-based one could greatly increase the facticity and accuracy of the predicted 3-D models of target proteins (Sokkar et al., 2011).

The AoXyn10 3-D model consists principally of the $(\beta/\alpha)_8$ barrel fold, which has been likened to a salad bowl (Fig. 4). One face of the AoXyn10 molecule has a larger radius (about 45 Å) due to an elaborate loop architecture, while the opposite face, which consists of simple α/β turns, has a radius of about 30 Å. This is similar to the fold described for family 5 enzymes, and both family enzymes are members of clan GH-A. Indeed, these two families are quite closely related and, in addition to sharing a common fold, they have the same type of catalytic mechanism and share several common residues (Larson et al., 2003). Two conserved glutamic acids in family 10 xylanases have been confirmed by site-directed mutagenesis to have a direct role in catalysis (Macleod et al., 1994). In this work, Glu^{131} and Glu^{238} are located in the hydrophobic cleft
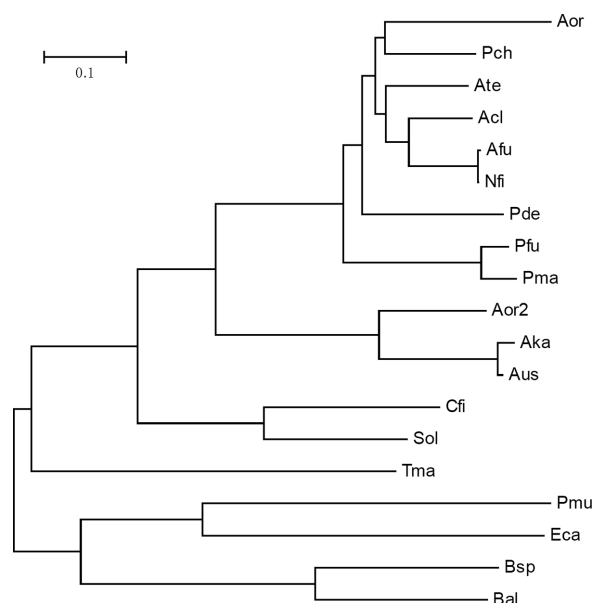
Fig. 3. A phylogenetic tree showing the evolutionary relativity and the homological degrees among family 10 xylanase sequences.

The estimated genetic distance between two sequences is in proportion to the length of a horizontal line connecting one sequence to another. The family 10 xylanase sequences are from the following microorganisms: Aor, *Aspergillus oryzae* CICC40186 (AFP43760, in this work); Pch, *Penicillium chrysogenum* Wisconsin 54-1255 (XP_002564127); Ate, *Aspergillus terreus* NIH2624 (XP_001212588); Acl, *Aspergillus clavatus* NRRL1 (XP_001268415); Afu, *Aspergillus fumigatus* Af293 (XP_751237); Nfi, *Neosartorya fischeri* NRRL181 (XP_001258500); Pde, *Penicillium decumbens* (ADX86896); Pfu, *Penicillium funiculosum* (CAG25554); Pma, *Penicillium marneffei* ATCC18224 (XP_002150978); Aor2, *Aspergillus oryzae* (BAB69073); Aka, *Aspergillus kawachii* (BAA03575); Aus, *Aspergillus usamii* (ADW20312); Cfi, *Cellulomonas fimi* (3CUF); Sol, *Streptomyces olivaceoviridis* E-86 (1V6Y); Tma, *Thermotoga maritima* (1VBR); Pmu, *Polyplastron multivesiculatum* (CAB65753); Eca, *Epidinium caudatum* (BAC57894); Bsp, *Bacillus* sp. (CAA84631); and Bal, *Bacillus alcalophilus* (AAQ99279).

of AoXyn10, where the β-1,4-xylosidic bond of xylan is inserted and is cleaved. There are two disulfide bridges in AoXyn10. One (Cys^{81}-Cys^{123}) connects strands β3 and β4, while the other (Cys^{266}-Cys^{272}) links helix α8 and strand β8 (Fig. 4).

Screening and expression of the transformants

P. pastoris transformants which could resist higher concentrations of G418 might have multi-copies of integration of the heterologous gene into the *P. pastoris* genome, which could potentially lead to a higher expression level of the heterologous protein as elucidat-
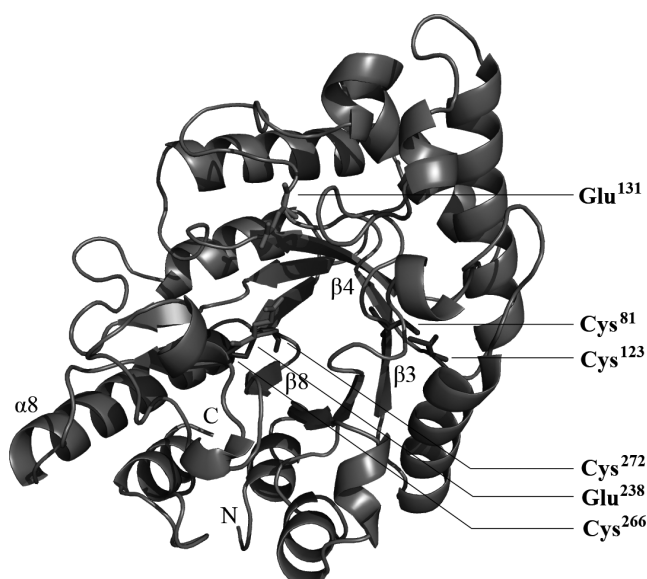
Fig. 4. The 3-D structure of the AoXyn10 CD predicted by multiple template-based homology modeling based on known crystal structures of family 10 xylanases from *Cellulomonas fimi* (3CUF), *Streptomyces olivaceoviridis* E-86 (1V6Y) and *Thermotoga maritima* (1VBR).

The catalytic residues, Glu[131] and Glu[238], are located in the hydrophobic cleft of AoXyn10. One disulfide bridge (Cys[81]-Cys[123]) connects strands β3 and β4, while the other (Cys[266]-Cys[272]) links helix α8 and strand β8.

ed in the manual of the Multi-Copy Pichia Expression Kit (Invitrogen). The protein expression level, however, was not directly proportional to the concentration of G418 (Li et al., 2011). For those reasons, we separately picked out 15 transformants resistant to 1.0, 2.0 and 4.0 mg/ml of geneticin G418, labeled as *P. pastoris* GS/Xyn1-1 to GS/Xyn1-15, GS/Xyn2-1 to GS/Xyn2-15 and GS/Xyn4-1 to GS/Xyn4-15, for flask expression tests. After a total of 45 *P. pastoris* transformants were induced by adding 2.0% (v/v) methanol at 24 h intervals for 96 h, the reAoXyn10 activity was measured. Among transformants tested, GS/Xyn4-11 with the highest reAoXyn10 activity of 45.0 U/ml was selected, while no xylanase activity was detected by the negative control (GSXC) under the same expression conditions.

Purification and N-deglycosylation of the expressed reAoXyn10

It was reported that the purities of recombinant *A. usamii* xylanase AuXyn11D and *A. sulphureus* β-mannanase expressed in *P. pastoris* GS115 and X-33 reached 90 and 97%, respectively (Chen et al., 2007; Zhang et al., 2012). In this work, the expressed reA-
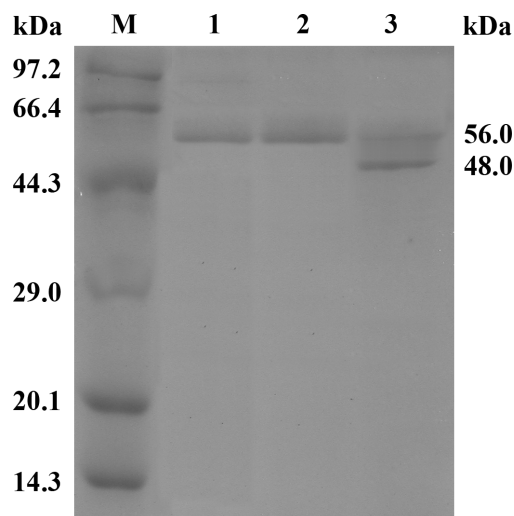
Fig. 5. SDS-PAGE analysis of the reAoXyn10 secreted by *P. pastoris* GS/Xyn4-11.

Lane M, the protein marker; lane 1, the cultured supernatant of GS/Xyn4-11; lanes 2–3, the purified reAoXyn10 and its N-deglycosylated protein with apparent molecular weights of about 56.0 and 48.0 kDa, respectively.

oXyn10 accounted for more than 88% of the total protein in the cultured supernatant, which was analyzed by protein band-scanning. Therefore, reAoXyn10 was purified to homogeneity only by a simple combination of ammonium sulfate precipitation, ultrafiltration and Sephadex G-75 gel chromatography (Fig. 5).

SDS-PAGE analysis of the purified reAoXyn10 displayed a single protein band with an apparent M.W. of about 56.0 kDa (Fig. 5, lane 2), which was much larger than the theoretical one (48,108 Da) of AoXyn10. *P. pastoris* enables some post-translational modifications including the assembly of a disulfide bond, exclusion of signal peptide and N-linked glycosylation. Analysis of the AoXyn10 sequence using the NetNGlyc program displayed three putative N-glycosylation sites (N[26], N[34] and N[101]). To verify whether the difference between apparent and theoretical M.W. resulted from N-glycosylation, the N-deglycosylation assay was performed. As a result, an additional protein band of about 48.0 kDa was observed on SDS-PAGE (Fig. 5, lane 3). Simultaneously, the carbohydrate content of the purified reAoXyn10 was determined to be 13.8%. Those experimental results verified that reAoXyn10 is an N-linked glycoprotein.

Enzymatic properties of reAoXyn10

The purified reAoXyn10 exhibited higher activity at a pH range of 4.5–6.0, between which the highest xyla-

nase activity was at pH 5.5 (measured at 50°C). Incubated at 40°C for 1 h at varied pH values (3.0−7.5), reAoXyn10 displayed high stability at a pH range of 4.0−7.0 (Fig. 6A). The temperature optimum of reAoXyn10 was 60°C (measured at pH 5.5). Incubated at pH 5.5 and at various temperatures (40−65°C) for 30 min, reAoXyn10 retained over 85% of its original activity at 50°C or below (Fig. 6B). The reAoXyn10 activity was not significantly affected by Fe^{2+}, Na^+, Al^{3+}, Zn^{2+}, Mg^{2+}, Cu^{2+}, Ca^{2+}, Co^{2+}, Sn^{2+}, Fe^{3+}, Li^+ or EDTA, but was inhibited by Mn^{2+} and Ba^{2+}. The K_m

and V_{max} of reAoXyn10, towards birchwood xylan at pH 5.5 and 60°C, were graphically determined from the Lineweaver-Burk plotting to be 1.7 mg/ml and 817 μmol/min/mg, respectively.

Our present work reports the cloning and analysis of the full-length cDNA and complete DNA sequences of *Aoxyn10*, and the homology modeling of the AoXyn10 CD. Moreover, *Aoxyn10* was extracellularly expressed in *P. pastoris* GS115 by an α-factor secretion signal. Enzymatic properties of the purified reAoXyn10 were characterized, revealing its low K_m and high V_{max}, broad pH stability, and strong resistance to most metal ions and EDTA. Considering the low thermostability of reAoXyn10, we will focus our further reserach on improving its thermostability by protein engineering, such as an increase in the number of salt bridges, and the introduction of disulfide bridges particularly at the N- or C-terminus or in the α-helix region.
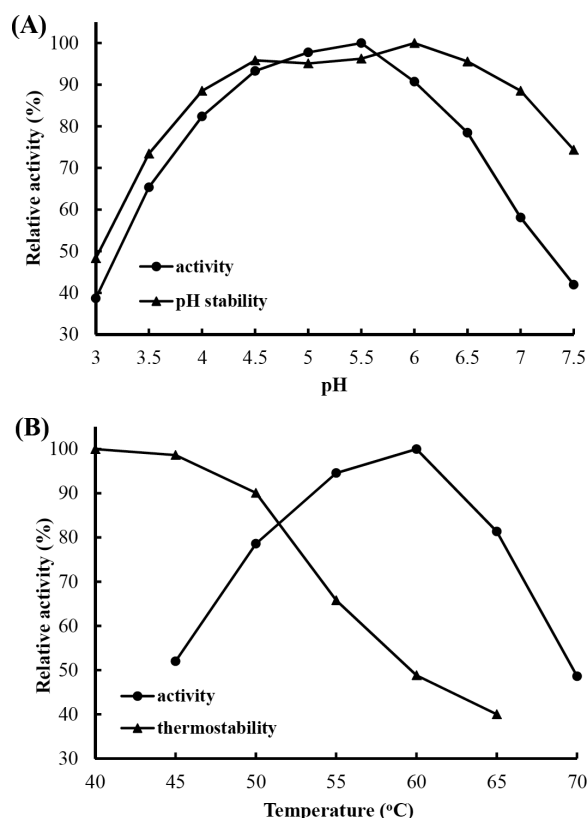

Fig. 6.   Effects of temperatures and pH values on the reAoXyn10 activity.
(A) pH optimum and stability. The pH optimum of the purified reAoXyn10 was measured under standard assay conditions, except for 0.5% (w/v) of birchwood xylan in 50 mᴍ Na_2HPO_4-citric acid buffer at a pH range of 3.0−7.5. The pH stability of reAoXyn10 was estimated by incubating it at 40°C for 1 h at varied pH values (3.0−7.5), and the residual activity of reAoXyn10 was measured under standard assay conditions. (B) Temperature optimum and stability. The temperature optimum of the purified reAoXyn10 was assayed, at pH optimum, at temperatures ranging from 45 to 70°C. The thermostability of reAoXyn10 was evaluated by incubating it for 30 min at different temperatures (40−65°C), and the residual activity of reAoXyn10 was measured under standard assay conditions.

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