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Cloning the gene encoding acetyl xylan esterase from *Aspergillus ficuum* and its expression in *Pichia pastoris*

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Abstract

Acetyl esterases are important in the complete degradation of acetylated polysaccharides, such as pectins and xylans. We isolated the gene encoding acetyl xylan esterase (AfAXE) from a genomic λ library of *Aspergillus ficuum*. We cloned the corresponding cDNA by RT-PCR. The *Afaxe* gene contained two introns, one TATAA box, and two CAAT-like boxes. The transcription initiation site was 61 bp upstream of the start codon. The deduced amino acid sequence consisted of a putative 28-amino acid leader peptide and a mature protein with an estimated molecular mass of 29.5 kDa. The nearest homolog of the cloned gene was acetyl xylan esterase of *A. niger*. The cloned gene was placed in a *Pichia* expression vector and expressed in *Pichia pastoris*. The culture filtrate of the transformant liberated acetyl moieties from *p*-nitrophenyl acetate and its activity reached 75.8 IU/ml, which was over 100-fold greater than the activity of the native enzyme expressed in *A. ficuum*. The cloned enzyme catalyzed the release of acetic acid from acetylated hardwood xylan, confirming that the cloned gene encoded an acetyl xylan esterase of *A. ficuum*. The native and recombinant acetyl xylan esterases were purified from the culture filtrates of *A. ficuum* and *P. pastoris*, respectively. Both enzymes had approximately the same optimal temperature (37 °C) and pH (7.0). The recombinant protein had greater tolerance for alkaline conditions (pH ≥ 7.0), but was less thermostable above 55 °C.

Keywords: *Aspergillus ficuum*; Acetyl xylan esterase; *Pichia pastoris*; Deacetylation

1. Introduction

Xylan, a polymer composed of 1,4-linked β-D-xylopyranosyl residues, is the most abundant hemicellulosic polysaccharide in plants [1]. Xylans are of structurally mixed composition and are highly decorated with non-xylose components, including *O*-acetyl, arbinose, ferulic acid, *p*-coumaric acid, and uronic acid moieties. These side groups hinder the complete degradation of hemicellulose by xalanase. The hardwood xylans are heavily acetylated at the C-3 position, and birchwood xylan has more than 1 mole of acetic acid per 2 moles of xylose [2].

Esterases are enzymes that cleave esterified substrates. Acetyl esterases (ACE; EC 3.1.1.6) are important in the complete degradation of acetylated polysaccharides, such as pectins and xylans. Accordingly, several cellulolytic and hemicellulolytic microbes contain enzymes specific for deacetylation. In addition to degrading esterified acetyl groups, some fungal acetyl esterases have substrate specificity for highly acetylated hardwood xylans; these fungal esterases are called acetyl xylan esterase (AXE) [3]. In addition to the complete degradation of acetylated xylan, AXEs, with combinations of other xylolytic enzymes, can cooperatively hydrolyze acetyl xylan and partially deacetylate acetyl xylan, which can be further processed by endo-xylanase to obtain oligomers of specific size [3].

AXEs have been purified from several fungi [4–10] and bacteria [11–14]. Moreover, the genes encoding AXE have been cloned from fungi [15–18] and bacteria [19–23]. Multiple forms of AXE are produced in *Trichoderma reesei*, while both ACE and AXE are produced simultaneously in *Aspergillus niger* [24].

AXE has several applications in industry and technology. Steam treatment of hardwood, as an initial step in separating cellulose from other components, produces a large soluble hemicellulose fraction containing xylan that, although not much valuable due to its heavy acetylation, is a lot more abundant and cheaper than softwood xylan. This can be used as chemical fuels for AXE yielding more digestible plant polysaccharide that could be used as animal feed [24], or as a substrate for the production of xylose or specific xylo-oligomers. Heterologous expression of AXE is desirable

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because it may provide a way to produce a large amount of enzyme. This enzyme could provide an enzymatic alternative to alkaline treatment for deacetylation. Pure AXE, uncontaminated by other xylolytic enzymes, could be used to pre-treat substrates for the production of xylo-oligomers of specific size. The methylotrophic yeast *Pichia pastoris* has been investigated as a promising high-yield production system for many heterologous proteins [25].

In this paper, we report the cloning of the gene encoding AXE (*Afaxe*) from *A. ficuum*, one of the most widely used fungi in industry. We report the heterologous expression of this gene in *P. pastoris*.

2. Materials and methods

2.1. Strains and culture conditions

Plasmids were maintained and propagated in *E. coli* TOP10F′ or DH5α according to Sambrook et al. [26]. *A. ficuum* (ATCC 16882) was incubated at 30 °C in complete medium containing 2% glucose, 2% malt extract, and 0.1% peptone. Induction medium for *A. ficuum* AXE was prepared as described in Linden et al. [27]. An Erlenmeyer flask containing 200 ml of medium was inoculated with 2×10^9 spores and incubated at 30 °C for 72 h in an orbital shaking incubator at 150 rpm.

P. pastoris GS115 (*his*4) was used as a recipient strain for *Afaxe* expression. *P. pastoris* was maintained on YPD plates (1% yeast extract, 2% peptone, 2% dextrose and 2% agar) at 30 °C.

2.2. Cloning and characterization of acetyl xylan esterase (Afaxe)

We designed degenerate primers specific for consensus nucleotide sequences from the most conserved regions within the catalytic domains of serine esterases. These primers were based on those used by Koseki et al. [28], with a few modifications. The primers used were *Afaxe*-F1 (forward, 5′-AGYTCRGGRGCYATGATG-3′) and *Afaxe*-R1 (reverse, 5′-AARCCRAACCACTCCAT-3′). Polymerase chain reaction (PCR) was conducted with 50 ng of fungal DNA and the following parameters: initial denaturation (94 °C, 4 min); followed by 30 cycles of denaturation (94 °C, 30 s), annealing (48 °C, 30 s), and extension (72 °C, 1 min); followed by one final cycle of 94 °C (30 s), 48 °C (30 s), and 72 °C (10 min). The 500-bp PCR product was cloned into pGEMT vector (Promega, Madison, WI, USA). The inserts of positive bacterial clones were sequenced and compared. One clone was selected and the insert was used as a hybridization probe for screening a genomic λ library of *A. ficuum* [26]. The genomic λ library of *A. ficuum* was constructed by ligating partially *Sau*3A1-digested genomic DNA into the λ replacing vector λDASHII cut with *Bam*HI (Stratagene, La Jolla, CA, USA).

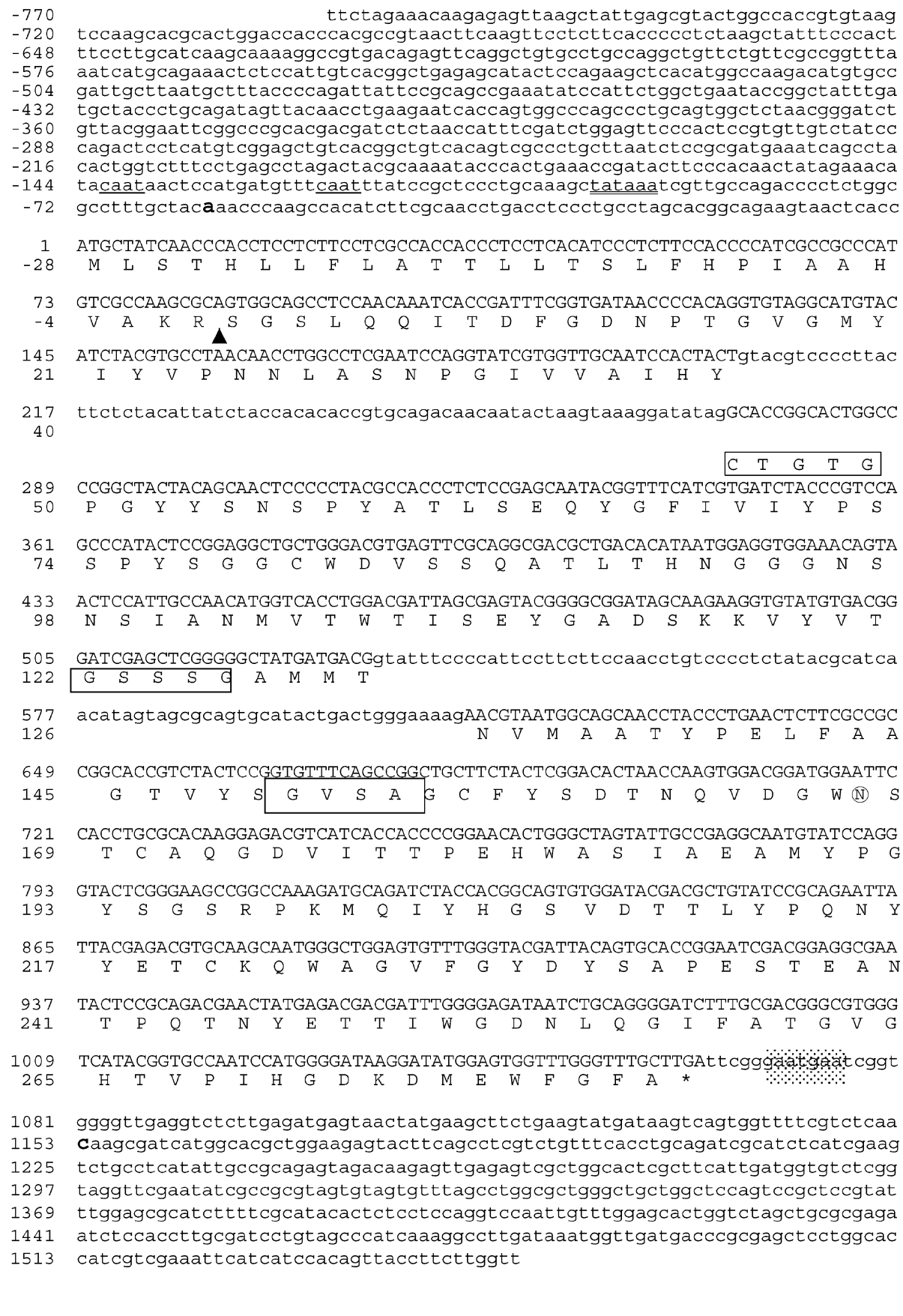
In order to obtain the cDNA clone of *Afaxe*, we performed PCR using reverse transcriptase (RT-PCR) with primers of *Afaxe*-F2 (forward, 5′-GAAGTAACTCACCATGC-3′) and *Afaxe*-R2 (reverse, 5′-CCCGAATCAAGCAAACCC-3′). The RT-PCR product was cloned and the near full-length cDNA clone was sequenced using synthetic oligonucleotide primers.

We conducted a primer extension experiment using a reverse primer (5′-GAAGAAGAGGTGTGATAGCATG-3′) to identify the transcriptional initiation site according to a standard procedure [26].

2.3. Heterologous expression of Afaxe in P. pastoris

A DNA fragment encoding the mature peptide of AXE was amplified from an *Afaxe* cDNA clone with two primers designed to generate *Cla*I and *Xba*I sites (forward, 5′-AATCGATTAGTGGCAGCCTCCAACAAATC-3′; reverse, 5′-TTCTAGAGCAAACCCAAACCATTCCATA-3′). The amplified fragment was digested with *Cla*I and *Xba*I, and inserted into the *Cla*I–*Xba*I-digested *P. pastoris* expression vector, pPICZαC (Invitrogen, Carlsbad, CA, USA). The resulting plasmid, pPIC-Axe, was used to transform *E. coli* TOP10, and transformants were selected on LB plates containing 25 μg/ml of Zeocin. The fusion of the mature peptide-encoding region of AXE with the yeast α factor at N-terminus and *myc* epitope followed by 6×His-tag tail at C-terminus was confirmed by DNA sequencing. A total of 10 μg of pPIC-Axe DNA was linearized by *Sac*I digestion and transformed into *Pichia* competent cells. The transformed cells were plated on YPD agar containing 100 μg/ml of Zeocin to screen for integration of the *Afaxe* gene into the 5′*AOX*1 region of the host chromosomal DNA. Each transformant was cultured in 100 ml of a buffered complex medium with glycerol (BMGY) for 24 h according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). After the culture reached a density of $OD_{600} = 6.0$, the cells were harvested, resuspended, and cultured in 100 ml of 0.5% methanol media (BMMY) to induce expression. The culture filtrates of positive clones were directly screened for AXE activity, and the secreted enzymes were monitored by SDS-PAGE at every 12 h for 72 h.

Fig. 1. Nucleotide and deduced amino acid sequence of *Afaxe* of *A. ficuum*. The intron and 5′- and 3′-flanking regions are shown in small letters. The two CAAT boxes are underlined, and the two TATA boxes are double underlined. The transcription initiation site and poly(A) site are shown in bold letters. The N-terminus of the putative mature protein is indicated by (▲). Two sites having the consensus motif of the active site serine enzyme and a unique N-glycosylation site are boxed and circled, respectively. The putative poly(A) signal is hatched. The GenBank accession number for the *Afaxe* sequence is AF331757.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-432 tgctaccctgcagatagttacaacctgaagaatcaccagtggcccagccctgcagtggctctaacgggatct
-360 gttacggaattcggcccgcacgacgatctctaaccatttcgatctggagttcccactccgtgttgtctatcc
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-144 tacaataactccatgatgtttcaattttatccgctccctgcaaagctataaatcgttgccagacccctctggc
-72 gcctttgctacaaacccaagccacatcttcgcaacctgacctccctgcctagcacggcagaagtaactcacc
1 ATGCTATCAACCCACCTCCTCTTCCTCGCCACCACCCTCCTCACATCCCTCTTCCACCCCATCGCCGCCCAT
-28 M L S T H L L F L A T T L L T S L F H P I A A H
73 GTCGCCAAGCGCAGTGGCAGCCTCCAACAAATCACCGATTTCGGTGATAACCCCACAGGTGTAGGCATGTAC
-4 V A K R S G S L Q Q I T D F G D N P T G V G M Y
145 ATCTACGTGCCTAACAACCTGGCCTCGAATCCAGGTATCGTGGTTGCAATCCACTACTgtacgtccccttac
21 I Y V P N N L A S N P G I V V A I H Y
217 ttctctacattatctaccacacaccgtgcagacaacaatactaagtaaaggatatagGCACCGGCACTGGCC
40
C T G T G
289 CCGGCTACTACAGCAACTCCCCCTACGCCACCCTCTCCGAGCAATACGGTTTCATCGTGATCTACCCGTCCA
50 P G Y Y S N S P Y A T L S E Q Y G F I V I Y P S
361 GCCCATACTCCGGAGGCTGCTGGGACGTGAGTTCGCAGGCGACGCTGACACATAATGGAGGTGGAAACAGTA
74 S P Y S G G C W D V S S Q A T L T H N G G G N S
433 ACTCCATTGCCAACATGGTCACCTGGACGATTAGCGAGTACGGGGCGGATAGCAAGAAGGTGTATGTGACGG
98 N S I A N M V T W T I S E Y G A D S K K V Y V T
505 GATCGAGCTCGGGGGCTATGATGACGgtatttccccattccttcttccaacctgtcccctctatacgcatca
122 G S S S G A M M T
577 acatagtagcgcagtgcatactgactgggaaaagAACGTAATGGCAGCAACCTACCCTGAACTCTTCGCCGC
126 N V M A A T Y P E L F A A
649 CGGCACCGTCTACTCCGGTGTTTCAGCCGGCTGCTTCTACTCGGACACTAACCAAGTGGACGGATGGAATTC
145 G T V Y S G V S A G C F Y S D T N Q V D G W Ⓝ S
721 CACCTGCGCACAAGGAGACGTCATCACCACCCCGGAACACTGGGCTAGTATTGCCGAGGCAATGTATCCAGG
169 T C A Q G D V I T T P E H W A S I A E A M Y P G
793 GTACTCGGGAAGCCGGCCAAAGATGCAGATCTACCACGGCAGTGTGGATACGACGCTGTATCCGCAGAATTA
193 Y S G S R P K M Q I Y H G S V D T T L Y P Q N Y
865 TTACGAGACGTGCAAGCAATGGGCTGGAGTGTTTGGGTACGATTACAGTGCACCGGAATCGACGGAGGCGAA
217 Y E T C K Q W A G V F G Y D Y S A P E S T E A N
937 TACTCCGCAGACGAACTATGAGACGACGATTTGGGGAGATAATCTGCAGGGGATCTTTGCGACGGGCGTGGG
241 T P Q T N Y E T T I W G D N L Q G I F A T G V G
1009 TCATACGGTGCCAATCCATGGGGATAAGGATATGGAGTGGTTTGGGTTTGCTTGAttcgggaatgaatcggt
265 H T V P I H G D K D M E W F G F A *
1081 ggggttgaggtctcttgagatgagtaactatgaagcttctgaagtatgataagtcagtggttttcgtctcaa
1153 caagcgatcatggcacgctggaagagtacttcagcctcgtctgtttcacctgcagatcgcatctcatcgaag
1225 tctgcctcatattgccgcagagtagacaagagttgagagtcgctggcactcgcttcattgatggtgtctcgg
1297 taggttcgaatatcgccgcgtagtgtagtgtttagcctggcgctgggctgctggctccagtccgctccgtat
1369 ttggagcgcatctttttcgcatacactctcctccaggtccaattgtttggagcactggtctagctgcgcgaga
1441 atctccaccttgcgatcctgtagcccatcaaaggccttgataaatggttgatgacccgcgagctcctggcac
1513 catcgtcgaaattcatcatccacagttaccttcttggtt

2.4. Enzyme purification

By virtue of the 6×His-tag at its C-terminus, the cloned enzyme was purified using a Ni–NTA agarose column chromatography according to the manufacturer's instructions (Qiagen, Chatsworth, CA, USA). At each step, the purified enzyme was evaluated by SDS-PAGE.

To isolate the native enzyme from *A. ficuum*, we followed procedures described previously [27,28]. The medium from an induced culture was concentrated by addition of ammonium sulfate to 80% of saturation. The resulting proteins were subjected to three successive columns: an anion exchange column of DEAE, a hydrophobic interaction column of Phenyl-5PW, and a gel-filtration column of G-200. Fractions of 3 ml each were collected and protein content was measured colorimetrically at 595 nm using the Bio-Rad protein reagent according to the method of Bradford [29] with bovine serum albumin as the standard protein.

2.5. Enzymatic assay

Cell growth and enzyme activity were measured for 3 days at 24-h intervals. Enzyme activity was measured from culture filtrate of recombinant yeast according to the modified protocol of Biely et al. [24]. Briefly, a solution of 0.05 g/ml of *p*-nitrophenyl acetate in 0.1 M potassium phosphate buffer (pH 7.0) was used as a substrate. The reaction was carried out at pH 6.5 and 37 °C. Absorption at 410 nm was observed at 20-s intervals over 3 min. The measurements were performed in triplicate and all experiments were carried out at least three times with similar results. The concentration of *p*-nitrophenol released by enzymatic reaction was determined from the standard curve obtained from the standard *p*-nitrophenol solution. One unit of enzyme is defined as the amount of enzyme needed to release 1 mmole of *p*-nitrophenol from its substrate, *p*-nitrophenyl acetate, per min at 37 °C.

In order to measure the thermal stability and pH optimum of the purified enzyme, the samples were pre-incubated for 1 h at appropriate temperature and pH, and then the residual activity was determined [28]. We measured thermostability between 10 and 80 °C with 5 °C increments. We measured the pH optimum from 5.0 to 8.5 with 0.5 increments.

In order to examine the glycosylation of the recombinant protein, we treated purified AfAXE with endoglycosidase H (endo-β-*N*-acetylglucosaminidase H of *Streptomyces plicatus*; Boehringer–Mannheim, Germany) according to the procedure of Trimble and Maley [30].

The substrate specificity of the cloned enzyme toward acetylated xylan was determined by measuring the released acetic acid from acetylated birchwood xylan according to the procedure described by Kormelink et al. [3]. The amount of acetate released was measured with a Hewlett Packard 1100 series high pressure liquid chromatograph equipped with an Aminex HPX 87H column maintained at 4 °C. The resulting peaks from acetic acid were detected with an HP 1315 diode array detector with a retention time of 12.2 min. Sodium acetate was used as a standard.

3. Results

3.1. Identification and characterization of the acetyl xylan esterase

Using degenerated primers, we obtained a 0.5-kb PCR product. The resulting nucleotide sequence showed the highest homology to the genes encoding acetyl xylan esterase of *A. niger* and acetyl esterase of *A. awamori* with 85 and 87% identities, respectively. The PCR product was then used to screen a genomic λ library of *A. ficuum*, and four out of 100,000 plaques showed positive results. A 2.5-kb *Xba*I–*Eco*RI-digested λ clone containing the acetyl xylan esterase gene (*Afaxe*) was selected for further analysis.

Based on the genomic sequence, we used RT-PCR to obtain the near-full length cDNA clone. The primers were located at nucleotide sequence −13 to +4 (forward) and +1068 to +1051 (reverse). By comparing the cDNA sequence with that of the genomic clone, we found that the *Afaxe* consisted of three exons with two intervening sequences of 71 and 80 bp, respectively (Fig. 1). Both introns contain canonical splicing donor and acceptor sequences. The internal conserved intron sequence, NCTRAC, was observed at 13 and 10 bp upstream of the 3′-end of each intron, respectively. Primer extension revealed that the transcription initiation site was at −61. In the promoter region of *Afaxe*, we found a canonical TATAAA and two CAAT-like boxes at −99, −123, and −142, respectively. The sequence around the first ATG was in good agreement with Kozak's consensus sequence in that the −3 position was A in CACCATG. The poly(A) site was observed 90 bp downstream of translational stop codon in the RT-PCR product and the putative poly(A) addition signal, AATGAA, was 7 bp downstream of the translational stop codon.

The deduced amino acid sequence of the cDNA was compared with those of the two most similar sequences: *A. niger* acetyl xylan esterase (AnAXE) and *A. awamori* acetyl esterase (AwACE) showing 92.7 and 91.9% similarity, respectively (Fig. 2). Both AnAXE and AwACE consist of 304 amino acid residues consisting of 29 amino acids of leader peptide and 275 amino acids of mature peptide. The sequence comparison reveals that the AfAXE has a putative 28-amino acid leader peptide which is presumably processed by the KEX2-like protease. The deduced AfAXE mature protein product consists of 275 amino acids with an estimated molecular mass of 29.5 kDa which is in a range similar to other AXEs: 30.5, 30.0, and 35.0 kDa for *A. niger*, *A. oryzae*, and *T. reesei*, respectively. The unique *N*-glycosylation site at Asn^{167} and two consensus sequences near the active site of the serine enzyme, G–X–S–X–G, are also well preserved. AfAXE is shorter by one amino acid in the leader peptide, and the missing residue appeared to be a

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AfAXE M-LSTHLLFLATTLLTSLFHPIAAHVAKRSGSLQQITDFGDNPTGVGMYIYVPNNLASNP 59
AnAXE *L*******VI**F****L***AA*AV********V********N*************** 60
AwACE *L*******VI**L****L***DG*AV********V********N*************** 60

AfAXE GIVVAIHYCTGTGPGYYSNSPYATLSEQYGFIVIYPSSPYSGGCWDVSSQATLTHNGGGN 119
AnAXE ******************SA****************************************** 120
AwACE ******************GD****************************************** 120

AfAXE SNSIANMVTWTISEYGADSKKVYVTGSSSGAMMTNVMAATYPELFAAGTVYSGVSAGCFY 179
AnAXE *************E*****S**F********L***************A************ 180
AwACE *************K*****S**F********M***************A************ 180

AfAXE SDTNQVDGWNSTCAQGDVITTPEHWASIAEAMYPGYSGSRPKMQIYHGSVDTTLYPQNYY 239
AnAXE *N****D*W************************S*******R******TLH********* 240
AwACE *N****V*L************************S*******R******SID********* 240

AfAXE ETCKQWAGVFGYDYSAPESTEANTPQTNYETTIWGDNLQGIFATGVGHTVPIHGDKDMEW 299
AnAXE ******S***********K*****************S********************** 300
AwACE ******A***********K*****************S********************** 300

AfAXE FGFA 303
AnAXE **** 304
AwACE **** 304
```

Fig. 2. Comparison of the deduced peptide sequences of acetyl xylan esterase from *A. ficuum* (AfAXE), acetyl xylan esterase from *A. niger* (AnAXE), and acetyl esterase from *A. awamori* (AwACE). Numbering of residues from the N-terminus of the whole protein is shown at the right. Identical amino acid residues are indicated by asterisks. Gap in alignment is indicated by a space.

Leu at either the second or third residue from the N-terminus of the signal peptide. In addition, there are a total of 28 amino acid residues that show deviations either between or among all three enzymes. Among all sequence deviations, most differences were in leader peptide.

3.2. Heterologous expression of Afaxe using P. pastoris

Afaxe was expressed in *P. pastoris* to examine whether the cloned gene encoded a functional enzyme. A total of 12 transformants of *P. pastoris* were selected on Zeocin medium and assayed for the secreted enzyme activity. After 12 h of induction, all recombinant strains reached to an early stationary phase and the culture filtrates of all transformants showed significant AXE activity. The enzyme activities increased further until 24 h after the induction. At this time, the enzyme activities was estimated to be 50–76 IU/ml of media (Fig. 3A). After 36 h induction, SDS-PAGE revealed that most of the secreted AfAXE had been cleaved into a slightly smaller fragment (Fig. 3B). However, there were no significant changes in the enzyme activity of the corresponding culture filtrate. The transformant exhibiting the highest AXE activity (76 IU/ml) was selected, cultivated until 24 h after the induction, and used for the enzyme purification.

3.3. Purification and characteristics of AfAXE

No enzyme activity was observed in the non-inducing complete medium of *A. ficuum*, which was consistent in the previous results of *Aspergillus* spp. [27,28]. However, using

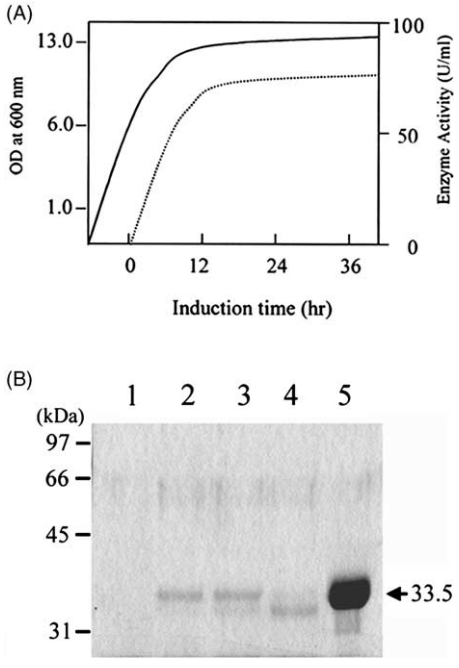

Fig. 3. Expression of *Afaxe* from recombinant *P. pastoris*. (A) Cell growth (solid line) and AXE enzyme activity (dashed line) in the culture filtrate of *P. pastoris* at various times after induction. (B) Coomassie blue stained SDS-PAGE of culture filtrates of *P. pastoris*. Samples of 10 ml were taken before induction (lane 1), after 12 h (lane 2), 24 h (lane 3), and 36 h (lane 4) of induction. For comparision, 5 ml of purified AfAXE from *P. pastoris* is shown (lane 5). Numbers on left refer to the estimated sizes (in kDa).

Table 1
Purification of acetyl xylan esterase

	Purifying steps	Total activity	Protein concentration (mg/100 ml)	Specific activity (IU/mg)	Purification fold	Recovery (%)
A. ficuum	Culture filtrate[a]	60	66.7	0.9	1	100
	DEAE-anion exchange	25.8	8.1	3.2	3.6	43
	Hydrophobic interaction	14.9	0.7	21.3	23.7	24.8
	Gel filtration	9.9	0.25	39.6	44.0	16.5
P. pastoris	Culture filtrate[a]	7580	527	14.4	1	100
	Ni-column	4548	140	32.5	2.2	60

[a] A total of 100 ml of induction medium, as described in Section 2 was used for the purification.

the culture filtrate of an inducing medium of *A. ficuum*, we obtained a 44-fold purification with a yield of 13% and a specific activity of 39.8 U/mg of protein. Using the culture filtrate of recombinant *P. pastoris*, we obtained a 2.2-fold purification with a yield of 60% and a specific activity of 32.5 U/mg of protein. The purification steps for both native and recombinant AXE are summarized in Table 1. Both

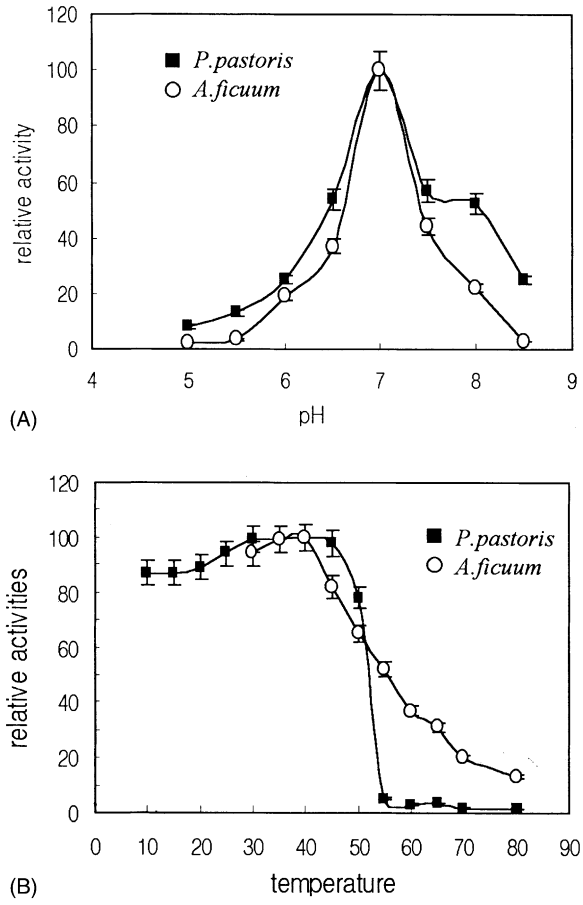

Fig. 4. Effects of pH (A) and temperature (B) on the activity of AfAXE. The native and recombinant enzyme activities are represented by open and closed marks, respectively. Data are shown as a representative profile based on two separate experiments, each with three samples for each data point.

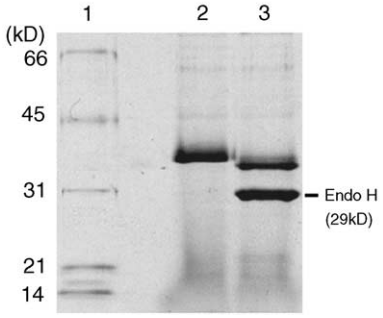

Fig. 5. SDS-PAGE of purified recombinant AfAXE (lane 2) and endoglycosidase H-treated AfAXE (lane 3). Protein size markers (lane 1) were used to estimate protein sizes (in kDa) (numbers at left).

enzymes had a pH optimum of 7.0, but the recombinant enzyme retained more activity above pH 7.0 (Fig. 4A). The thermal stability of both enzymes decreased at temperatures above 40 °C. The recombinant enzyme was more labile to thermal inactivation: above 55 °C all recombinant enzyme activity was lost, while the native enzyme maintained some residual activity to 80 °C (Fig. 4B).

We examined the glycosylation of the purified AfAXE enzyme. Treatment with endoglycosidase H changed the 36-kDa band into a smaller band at 34 kDa with a concomitant endoglycosidase H at 29 kDa (Fig. 5). Thus, AfAXE appears to be glycosylated in *P. pastoris*.

We examined the substrate specificity of the yeast-expressed enzyme to see whether the enzyme preparation was able to release acetic acid from steamed birchwood xylan. After the incubation of the recombinant culture filtrate with birchwood xylan, acetic acid was detected by HPLC within 1 h whereas no acetic acid was observed using the culture filtrate of the host strain of *P. pastoris* (data not shown).

4. Discussion

The structure and organization of *Afaxe*, including the TATAA, CAAT, intron-splicing sites, and poly(A) signal are all typical of filamentous fungi [31]. The *Afaxe* gene, along with the closely related *Anaxe* and *Awace* genes, contains

two CAAT boxes in its promoter. No other known acetyl xylan esterases, however, contain two CAAT-like sequences.

It is possible that the cloned gene in this study may encode an acetyl esterase that is not an acetyl xylan esterase. Indeed, *A. niger* produces both AXE and ACE activities, so it is possible that *A. ficuum* contains both as well. One of the closest homologues of the gene we cloned is the ACE of *A. awamori*. However, the strong induction of a native enzyme by xylan substrate and the liberation of acetic acid from steamed birchwood xylan by the heterologously expressed gene product strongly indicate that the cloned gene indeed encodes a functional AXE of *A. ficuum*.

The acetyl esterase activity in the culture filtrate of *A. ficuum* was 0.6 IU/ml, which is in a similar order of magnitude as those reported for *A. awamori* (1.0 IU/ml) [28] and *A. niger* (1.9 IU/ml) [27]. Kormelink et al. [3] reported an activity of 55 IU/ml for *A. niger* AXE. The discrepancy between the Kormelink and Linden studies seems to be due to the use of different strains and/or reaction conditions.

The enzyme activity we found in the culture filtrate of recombinant yeast was 75.8 IU/ml, more than 100-fold greater than that of *A. ficuum*. The specific activity of the recombinant enzyme was also greater: 14.4 IU/mg for the recombinant enzyme versus 0.9 IU/mg for *A. ficuum*. This productivity is believed to be the highest yield of bioactive AXE with a considerable purity in the culture filtrate. Based on the time course study of AfAXE expression, most of secreted AfAXE started to be cleaved into a smaller, bioactive fragment after 24 h of induction, which suggests that a site-specific extracellular protease(s) is involved in the proteolysis of AfAXE. Moreover, during the purification of AfAXE on Ni–NTA resin, most of AfAXE activity from the longer cultures (>36 h) was found in the wash buffer instead of the elution buffer, which suggests that the specific cleavage site lies between the active site and the C-terminus of the protein.

Compared to the native enzyme, the purified recombinant AXE showed an improved tolerance for pHs ranging from 7.5 to 8.5, but a reduced thermal stability above 55 °C. The discrepancies between the native and recombinant enzymes seem to be due to different post-translational modification(s). Compared to baker's yeast, *Saccharomyces cerevisiae*, proteins secreted by *Pichia* may have an advantage, because they are not hyperglycosylated [32]. Our purified AfAXE appeared to contain a unique canonical N-linked glycosylation sites (Asn–X–Ser/Thr) at Asn-167. Treatment with endoglycosidase H treatment clearly demonstrated that AfAXE is a glycosylated protein (Fig. 5). This glycosylation may result in the different characteristics of recombinant AXE. In addition to the glycosylation, other factors, such as the extra C-terminal tag residues, may affect the characteristics of the enzyme.

The present study has reported the cloning of a gene encoding an acetyl xylan esterase from *A. ficuum*, its expression in *P. pastoris*, and a comparison of the characteristics of the native and recombinant enzymes. Heterologous expression of AXE in this study makes it possible to over-produce the AXE without any contamination of other xylolytic enzymes. This enzyme offers the possibility of an enzymatic alternative to chemical deacetylation and a better method for controlled production of xylo-oligomers of specific sizes.

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