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ORIGINAL PAPER

Heterologous expression and biochemical characterization of acetyl xylan esterase from *Coprinopsis cinerea*

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Abstract Acetyl xylan esterase (AXE) from basidiomycete *Coprinopsis cinerea* Okayama 7 (#130) was functionally expressed in *Pichia pastoris* with a C-terminal tag under the alcohol oxidase 1 (AOX1) promoter and secreted into the medium at 1.5 mg l^{-1}. Its molecular mass was estimated to be 65.5 kDa based on the SDS-PAGE analysis, which is higher than the calculated molecular mass of 40 kDa based on amino acid composition. In-silico analysis of the amino acid sequence predicted two potential N-glycosylation sites. Results from PNGase F deglycosylation and mass spectrum confirmed the presence of N-glycosylation on the recombinant AXE with predominant *N*-glycans $HexNAc_2Hex_{9-16}$. The recombinant AXE showed best activity at 40 °C and pH 8. It showed not only acetyl esterase activity with a K_m of 4.3 mM and a V_{max} of 2.15 U mg^{-1} for hydrolysis of 4-nitrophenyl acetate but also a butyl esterase activity for hydrolysis of 4-nitrophenyl butyrate with a K_m of 0.11 mM and V_{max} of 0.78 U mg^{-1}. The presence of two additional amino acid residues at its native N-terminus was found to help stabilize the enzyme against the protease cleavages without affecting its activity.

Keywords *Coprinopsis cinerea* · Acetyl xylan esterase (AXE) · *Pichia pastoris* · Expression · Glycosylation · Enzyme activity

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Introduction

Plant biomass is enriched with carbohydrate polysaccharides. Plant cell wall predominantly contains cellulose, hemicellulose and lignin. The carbohydrate polymers cellulose and hemicellulose are interlinked with aromatic polymer lignin (Juturu and Wu 2012). Hemicellulose is a branched heteropolymer with xylan being the significant component in hardwood and herbaceous biomass. Xylan is composed of xylose units which are interconnected by 1, 4-linked β-D-xylopyranose bonds. The xylan backbone is randomly substituted with arabinose, mannose, galactose, ferulic acid, acetic acid and glucuronic acids by means of glycosidic and ester bonds (Kumar et al. 2008). The complete depolymerization of hemicellulose polymer requires synergistic actions of various hemicellulases, which are classified into glycosyl hydrolases (GH) that hydrolyze glycosidic bonds and carbohydrate esterases (CE) that act on the ester bonds between acetic or ferulic acid substitution and xylan backbone (Shallom and shoham 2003). Hemicellulases include exo-xylanase (exo-1, 4-β-xylanase, E.C.3.2.1.37), endo-xylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan-1,4-β-xylosidase, E.C.3.2.1.37), α-glucuronidase (α-glucosiduronase, E.C.3.2.1.139), α-arabinofuranosidase (α-L-arabinofuranosidase, E.C.3.2.1.55), feruloyl xylan esterases (EC 3.1.1.73) and acetyl xylan esterase (E.C.3.1.1.72) (Juturu and Wu 2012).

Three xylanases, exo-xylanase, endo-xylanase and β-xylosidase, are responsible for the complete hydrolysis of xylan to xylose. However, the acetylation of xylan has been reported to inhibit the action of exo-xylanase reducing the xylose yield. The combined use of xylanases and acetyl xylan esterase (AXE) has been shown to enhance the hydrolysis of xylan (Biely et al. 1986). In addition, AXEs may also be useful in catalyzing the reverse reaction of

acetyl bond hydrolysis for synthesis of useful compounds in organic solvents (Klibanov 2001). AXEs are classified into the CE families 1, 2, 3, 4, 5, 6, 7, 12 and 16 according to CAZY database (www.cazy.org) and produced by a wide variety of microorganisms in nature. Among them, fungal AXEs are commercially important because of their high activity, good stability and broader substrate diversity as a result of their diverse inhabitant environments in nature. The coding sequences of AXEs from *Aspergillus aculeatus* CBS 101.43, *Humicola insolens* DSM 1800 and *Thielavia terrestris* NRRL 8126 have been patented (Christgau et al. 2000; Marantha and Brown 2010; Lopez and Ding 2010). Expression of AXE genes in suitable hosts for large scale production is of great importance in reducing the enzyme production cost for commercial applications. Methylotrophic yeast *Pichia pastoris* has been recognized as an excellent host for expressing a wide range of heterologous proteins owing to its high expression level and mature functions of eukaryotic posttranslational modifications such as disulfide bond formation and glycosylation (Macauley-Patrick et al. 2005). Until now, only a few fungal AXEs from *Aspergillus awamori*, *Thermobifida fusca* and *Volvariella volvacea* have been functionally expressed in *P. pastoris* (Koseki et al. 2005; Yang et al. 2010; Tian et al. 2012).

Here we report the functional expression of the AXE from *Coprinopsis cinerea* Okayama 7 (#130) in *P. pastoris* and characterization of the recombinant enzyme. *C. cinerea* Okayama 7 (#130) is a multicellular basidiomycete with a typical mushroom form that undergoes a complete sexual cycle. Unlike most mushrooms, *C. cinereus* can complete its entire life cycle in laboratory, which may indicate its strong ability of metabolizing lignocellulose with highly active hydrolytic enzymes including AXE that may be used in lignocelluloses-based biorefinery.

Materials and methods

AXE nucleotide sequence

The cDNA of *C. cinerea* Okayama 7 (#130) AXE coding sequence (accession number XM001840041.1) was obtained from the nucleotide database of NCBI (Stajich et al. 2010). It has 1,125 bp encoding a polypeptide of 374 amino acids with a molecular mass of 39.66 kDa. *In silico* analysis predicts a native signal peptide consisting of 22 amino acid residues.

Construction of yeast expression plasmid

The synthetic gene of AXE was ligated into plasmid pUC57 between restriction sites *Xho*I and *Xba*I, which was used as a template to get the enzyme coding sequence (CDS) without the native signal peptide sequence by PCR for cloning into the yeast plasmid pPICZα A in two different constructs for secretory expression (Genescript, NJ, USA). For the first construct, the CDS was cloned immediately after the Kex2 and Ste 13 cleavage sites using the restriction sites *Xho*I and *Xba*I (the plasmid was named as pPICZαA-AXE (*Xho*I)) to get the recombinant protein with a native N-terminus. For the second construct, the CDS was cloned between the *EcoR*I and *Xba*I sites within the multiple cloning sites (the plasmid was named as pPICZαA-AXE (*EcoR*I)) to get the recombinant protein with two additional amino acids Glu and Phe at its native N-terminus. For both constructs, a polyhistidine tag (6×) was inserted before the stop codon at the C-terminus of the protein during subcloning to facilitate the protein purification. The molecular masses of the two recombinant proteins were estimated to be 40.5 and 40.77 kDa, respectively, before post translational modifications.

Gene transformation and clone selection

The yeast expression plasmids were amplified in *Escherichia coli* top 10. The plasmid DNA was isolated from the bacterial cells using the Qiagen Miniprep Kit and linearized in the 5′ AOXI region using *Sac*I restriction enzyme to facilitate its insertion into the AOX1 locus of *P. pastoris*. The linearized plasmid DNA was purified over a PCR purification column and eluted using 10 µl of elution buffer to yield a concentrated DNA for yeast transformation. The electrocompetent cells of *P. pastoris* KM71 were prepared just before the transformation as instructed by the supplier. The yeast cells were harvested at $OD_{600} = 1$, washed with sterile ice-cold water and 1 M sorbitol sequentially, and then resuspended in 1 ml of 1 M ice-cold sorbitol for transformation use.

The linearized plasmid DNA (6.0 µg) was transformed into 50 µl yeast competent cells using a Micro Pulser (Bio-Rad, Hercules, CA) with a pre-set program PIC (1.99 kV, 6.10 ms) specific for *P. pastoris.* The transformants were regenerated in 2 ml ice-cold 1 M YPS (1 % yeast extract, 2 % peptone and 1 M sorbitol) at 30 °C for 2 h without shaking. Afterwards, 200 µl portions of the regeneration culture were plated onto the YPDS (1 % yeast extract, 2 % peptone, 2 % dextrose, 1 M sorbitol and 2 % agar) plates containing 100 µg ml^{-1}zeocin.

Expression of AXE by fermentation in shaking flasks

Few transformant colonies were randomly picked up and inoculated separately into a 250 ml baffled conical flasks containing 50 ml of YPD medium supplemented with 100 µg ml^{-1} zeocin. The flasks were incubated at 28 °C

and 250 rpm for 48 h. The primary induction of the stationary phase cell cultures (50 ml) was done by mixing with 50 ml of minimal medium with methanol (1 %, v/v) for 8 h, followed by addition of 1 % (v/v) of absolute methanol and incubation for another 16 h. Then 0.5 % (v/v) of absolute methanol was supplemented and the culture was continued for 8 h followed by addition of 1 % (v/v) of absolute methanol for further cultivation for 16 h. The above induction operation was repeated for another 3 days. After induction phase, yeast cultures were left un-induced for 2 days to detect the proteolytic cleavage of the secreted AXE. During the induction period, 10 ml aliquots of yeast cultures were collected every 24 h and 10 ml of minimal medium without methanol was supplemented into the culture at the same time to reimburse the depleted volume followed by immediate methanol induction. The collected yeast cultures (10 ml) were centrifuged at 4 °C and 4,000 rpm for 10 min. The supernatants were collected in new tubes and kept on ice for various analyses.

Purification of AXE and gel electrophoresis

The aerobic fermentation culture was centrifuged to remove yeast cells from the broth. The broth containing recombinant AXE was used for protein purification using HiTrap Chelating HP column (GE Healthcare, Uppsala, Sweden) following the supplier's protocol. The primary column was made ready for protein purification by loading 0.1 M $NiSO_4$ followed by washing the column with deionized water to remove the excess $NiSO_4$. The column was then equilibrated with the binding buffer without imidazole (0.02 M sodium phosphate, 0.5 M NaCl, pH 7.4). Then 10 ml broth was loaded onto the column followed by washing with 5 ml binding buffer to remove undesired proteins. The enzyme bound to the column was eluted using 5 ml elution buffer (0.02 M sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4). The purity of the eluted enzyme (0. 15 mg l^{-1}) was verified over the NuPAGE Novex Tris–Acetate Mini Gels (Invitrogen, Carlsbad, USA).

Protein concentration and activity assays

The concentration of purified AXE was estimated by Bradford method using bovine serum albumin (BSA) as the standard. 4-Nitrophenyl acetate (4-NPA) and 4-nitrophenyl-butyrate (4-NPB) were used as the substrates to detect the esterase activities, respectively. The activity assay was performed by incubating 0.9 ml of 2 mM substrate in 100 mM phosphate citrate buffer (pH 7) with 100 µl purified enzyme at 40 °C for 10 min. The enzymatic reaction was stopped by adding 0.75 ml of 1 M Na_2CO_3. The release of 4-nitrophenol was measured at 405 nm using a plate reader. One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 µmol of 4-nitrophenyl acetate (4-NP) in 1 min.

Determination of optimal conditions for enzyme activity

The effect of pH on enzyme activity was investigated at pH 3–9 using phosphate citrate buffers (100 mM, pH 3–6) or sodium phosphate buffers (100 mM, pH 7–9) to get the desired pHs.

Optimal temperature was investigated by assaying the enzyme activity in 100 mM sodium phosphate buffer (pH 8) at 30–80 °C. The Michaelis–Menten kinetic constants were obtained at pH 8 and 40 °C using 4-nitrophenyl acetate (4-NPA) and 4-nitrophenyl butyrate (4-NPB) as the substrates, respectively. The K_m and V_{max} values were calculated from the Lineweaver–Burk plots using the Graphpad Prism software (GraphPad Software, Inc, California, USA).

Preparation of acetylated xylan

Acetylated xylan was prepared following the method of Belmokaddem et al. (2011). Xylan powder (1 g) was dispersed in 2.5 ml glacial acetic acid with stirring and heated to 75 °C for 5 min. Then 1.8 ml acetic anhydride and 150 µl methanesulfonic acid were added and the mixture was kept at 75 °C for 1 h, followed by addition of 10 ml absolute ethanol to stop the acetylation reaction. The precipitated xylan was collected by centrifugation at 4,000 rpm, 4 °C for 10 min, washed with 25 ml 70 % ethanol by thorough vortexing, and collected by centrifugation at 4,000 rpm, 4 °C for 10 min. This washing step was repeated for 7 times. The finally collected xylan was resuspended in 25 ml double distilled water and frozen at −80 °C overnight followed by lyophilization to get dry powder of acetylated xylan.

The acetylation degree of xylan was determined by alkaline hydrolysis. Acetylated xylan (1 mg) was dissolved in 1 ml double distilled water. To this solution was added 2 ml of 2 M NaOH. The reaction mixture was incubated at 50 °C overnight followed by centrifugation at 6,000 rpm for 10 min. The supernatant was collected after passing through a membrane (pore size 0.22 µm) and analyzed by HPLC (Shimadzu, Japan) with an Aminex HPX-87H column (300 mm × 7.8 mm) at 50 °C to detect the released acetic acid. Samples (50 µl) were eluted by 0.005 M H_2SO_4 at 0.65 ml min^{-1} and detected by a refractive index detector.

Glycosylation analysis for release of *N*-glycans by PNGase F treatment

Purified AXE (10 µg) was resuspended in 20 µl 5 mM NH_4HCO_3 followed by addition of 0.5 µl Peptide

N-Glycosidase F (PNGase F) (*Flavobacterium meningosepticum*; New England Biolabs). The mixture was incubated at 37 °C overnight. The reaction was stopped by freezing followed by drying the sample in a vacuum centrifuge at 40 °C. BondElut-C18 column (Agilent, USA) was activated with 1 ml 80 % acetonitrile (ACN)/0.1 % triflouracetic acid (TFA) and equilibrated with 2 ml 0.1 % TFA. The dried sample was resuspended in 100 µl of 0.1 % TFA and applied onto the column. The purified *N*-glycans were eluted with 0.9 ml of 0.1 % TFA into a glass vial.

To estimate the molecular mass of de-(*N*)-glycosylated AXE, 10 µg of pure enzyme was mixed with 0.5 µl PNGase F and the mixture was incubated at 37 °C overnight. The enzyme was precipitated using methanol/chloroform as follows. Into the protein solution was added 4 volumes of methanol, 3 volumes of distilled water and 1 volume of chloroform. The mixture was centrifuged at 13,000 rpm for 5 min. The liquid phase was decanted and the precipitated protein was washed with 4 volumes of methanol followed by centrifugation at 13,000 rpm for 10 min and drying in a fume hood. The protein pellet was resuspended in 20 µl SDS-loading dye and applied onto a 12.5 % SDS-gel along with 10 µg of untreated AXE.

Glycosylation analysis for release of O-glycans by reductive β-elimination

De-(*N*)-glycosylated AXE was used to detect the presence of potential O-glycans. The de-(*N*)-glycosylated protein band from the protein gel was excised from Coomassie-stained gel and washed with 1 ml double distilled water for 1 h followed by washing with 1 ml 50 % acetonitrile twice. The gel slice was dried in a vacuum centrifuge at 40 °C and equilibrated with 1 ml of 50 mM NH_4HCO_3 buffer (Pronase buffer) for 1 h and dried by vacuum centrifugation. Protease enzyme Pronase E (2 µg) in 100 µl Pronase buffer was added to the gel slice to start the digestion at 37 °C overnight. The released peptides were eluted in 200 µl double distilled water and 200 µl 50 % acetonitrile, respectively, each for 1 h. The eluents were combined and dried by vacuum centrifugation. O-Glycans if any would be released from the glycopeptides by reductive β-elimination. The dried sample was incubated with 0.5 M $NaBH_4$ in 100 mM NaOH at 50 °C overnight. The reaction was stopped by adding 2 µl of 1 M acetic acid. The sample was desalted in 50 µl Dowex 50WX8 (Bio-Rad, Germany, Munich) incubated on a shaker at 25 °C for 5 min. Then the mixture was centrifuged at 13,000 rpm for 1 min, the supernatant was collected into a new glass vial. The desalted samples were washed with 50 µl double distilled water and treated as described above. The samples were dried by vacuum centrifugation at 40 °C.

Permethylation of glycans

Permethylation of glycans was performed under inert conditions in an argon atmosphere following the method of Ciucanu and Kerek (1984). To the glycan samples were added 20 µl dimethyl sulfoxide (DMSO) and 40 µl of 1 M NaOH in DMSO (Anumula and Taylor 1992). The mixture was incubated at room temperature for 30 min followed by addition of 25 µl CH_3I to start the permethylation reaction, which was stopped after 30 min by adding 40 µl of 1 M acetic acid. Then 300 µl $CHCl_3$ and 200 µl double distilled water were added and the mixture was centrifuged at $5{,}000 \times g$ for 1 min to get the permethylated glycans. The aqueous phase was decanted and 200 µl fresh double distilled water was added. The sample was centrifuged to remove the aqueous phase. This washing step was repeated 3 times. The glycans obtained were dried under N_2 (Ciucanu and Kerek 1984).

Mass spectrum analysis of glycans by MALDI-TOF

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on an ultraflextreme instrument (Bruker Daltonics). The permethylated glycans were resuspended in 10 µl methanol and 0.5 µl aliquots were mixed with 1 µl of matrix (A saturated solution of 2,5-dihydroxy benzoic acid in 80 % acetonitrile). The samples were analyzed by detection in positive ion reflectron mode. Ionization of co-crystallized glycans was induced with a pulsed smart-beam laser and the ions were accelerated at 20 kV and reflected at 23 kV.

Activity assay with acetylated xylan

Acetylated xylan (2 %, w/v) in 10 ml phosphate–citrate buffer (100 mM, pH 7) was mixed with 4 µg ml^{-1} purified AXE and incubated at 40 °C, 200 rpm for 24 h. The deacetylation reaction was stopped by heating the mixture at 95 °C for 5 min. The mixture was centrifuged at 13,000 rpm for 5 min. The supernatant was collected after passing through a membrane (pore size of 0.22 µm). The released acetic acid was analyzed by HPLC as described above.

Results

Characterization and sequence analysis of AXE

The complete genome sequence of *C. cinerea* has been published in the nucleotide data base, but the AXE gene coding sequence was only annotated with no additional details provided (Stajich et al. 2010). The total length of

the AXE gene is 1,125 bp encoding a peptide chain of 374 amino acids. The peptide chain has a putative signal peptide of 22 amino acids long at the N-terminus (http://www.cbs.dtu.dk/services/SignalP/). The signal peptide is highly hydrophobic with 16 nonpolar/apolar amino acids. The mature protein contains a conserved N-terminal cellulose-binding module (CBM 1) which is linked to a catalytic domain belonging to the esterase–lipase super family. Two potential N-glycosylation sites were predicted at the asparagine positions 114 and 320 flanking the esterase domain (http://www.cbs.dtu.dk/services/NetNGlyc/). *In-silico* analysis of the peptide sequence indicates the presence of 8 cysteines with the possibility of forming 2 disulfide bridges between residues Cys^{40}–Cys^{46} and Cys^{138}–Cys^{175} at high scores of 0.96 and 0.97 (http://clavius.bc.edu/~clotelab/DiANNA/), respectively. This enzyme contains a conserved Ser^{219} in the consensus sequence typical for serine esterases: Gly–Thr–Ser–Ser–Gly. This motif is highly conserved within AXEs (Fig. 1).

Blast search of the amino acid sequence of this enzyme with the protein data base shows its high sequence similarity with other AXEs from *Piriformospora indica* (66 %, accession no. CCA73570.1), *Penicillium purpurogenum* (65 %, accession no. AF529173), *V. volvacea* (63 %, accession no. DQ888226) and ferulic acid esterase B from *Penicillium funiculosum* (55 %, accession no. AJ291496) as well as other esterases such as the esterase from *Glomerella graminicola* (56 %, accession EFQ27328.1) and a CE1 family esterase from *Botryotinia fuckeliana* (61 %, accession no. CCD475 23.1) (Fig. 2).

Gene transformation, expression and protein purification

Pichia pastoris KM71 transformants carrying the two types of AXE expression plasmids showed clear colonies on YPDS plates with 100 μg ml^{-1} zeocin after 2 days' incubation at 28 °C. The randomly picked up transformants were cultivated in shaking flasks to compare their expression levels of the recombinant protein. The yeast clones bearing either of the two expression plasmids started to express the recombinant protein after 1 day's induction and reached maximal expression on day 2. However, the expression level of the AXE with two additional amino acids at its native N-terminus maintained almost constant since day 3 until day 7. In contrast, the expression level of the AXE with its N-terminus started to decrease from day 3 and the protein was completely degraded by day 7 (Fig. 3). Two transformants bearing the plasmids pPICZαA-AXE (*Xho*I) and pPICZαA-AXE (*Eco*RI) were found to yield the highest protein expression levels at 1.3 and 1.5 mg l^{-1}, respectively.

Characterization of recombinant AXE

The purified AXEs with and without the two amino acids in its native N-terminus showed a clear diffused band on the SDS-PAGE with an estimated molecular mass of 65.5 kDa, which is 25.0 kDa higher than the predicted 40.5 kDa prior to post-translational modifications. The enzymes showed their maximal esterase activity at pH 8.0 (Fig. 4) and 40 °C (Fig. 5). The K_m and V_{max} under the optimal conditions were determined to be 4.3 mM and 2.15 U mg^{-1} for 4-NPA, and 0.11 mM and 0.78 U mg^{-1} for 4-NPB, respectively, irrelevant with the existence or not of the two additional amino acids in its N-terminus of the native protein.

The acetylation degree of the acetylated xylan was determined to be 43 % (w/w). Incubation of acetylated xylan (2 %) with 40 μg purified AXE in 10 ml sodium phosphate buffer (100 mM, pH 8) at 40 °C for 24 h released 1.8 g l^{-1} of acetic acid.

Glycosylation of AXE

The de-(*N*)-glycosylated AXE by treatment with PNGase F showed a reduced molecular mass of 35 kDa (Fig. 6). The MALDI-TOF mass spectrometry analysis of the permethylated de-(N)-glycosylated AXE showed 8 peak groups separated by an interval of 204 kDa. The mass difference resembles the addition of a hexose residue to the glycan chain. The identified oligosaccharides were specific for $HexNAc_2Hex_{9–15}$ with $HexNAc_2Hex_{10}$ being the most prominent chain (Fig. 7). No obvious peaks coinciding with O-glycosylation were observed from the mass spectrum.

Discussion

The AXE from basidiomycete *C. cinerea* was functionally expressed in the secretory mode in methylotrophic yeast *P. pastoris* either in the form with its native N-terminus or in the form with two additional amino acids at its native N-terminus. The purified AXE showed a higher-than-predicted molecular mass due to the N-glycosylation, which has been elucidated to be $HexNAc_2Hex_{9-16}$ based on the analyses of the enzymatic deglycosylation and mass spectrum analysis of glycans. No O-glycosylation was detected in the recombinant AXE. N-glycosylation seems to be a common feature of recombinant fungal AXEs expressed in *P. pastoris* so far (Koseki et al. 2006, Yang et al. 2010, Tian et al. 2012). The N-glycosylation of AXE from *C. cinerea* in *P. pastoris* was confirmed to be $HexNAc_2Hex_{9–15}$ with $HexNAc_2Hex_{10}$ being the most prominent chain, which is in agreement with the general concept

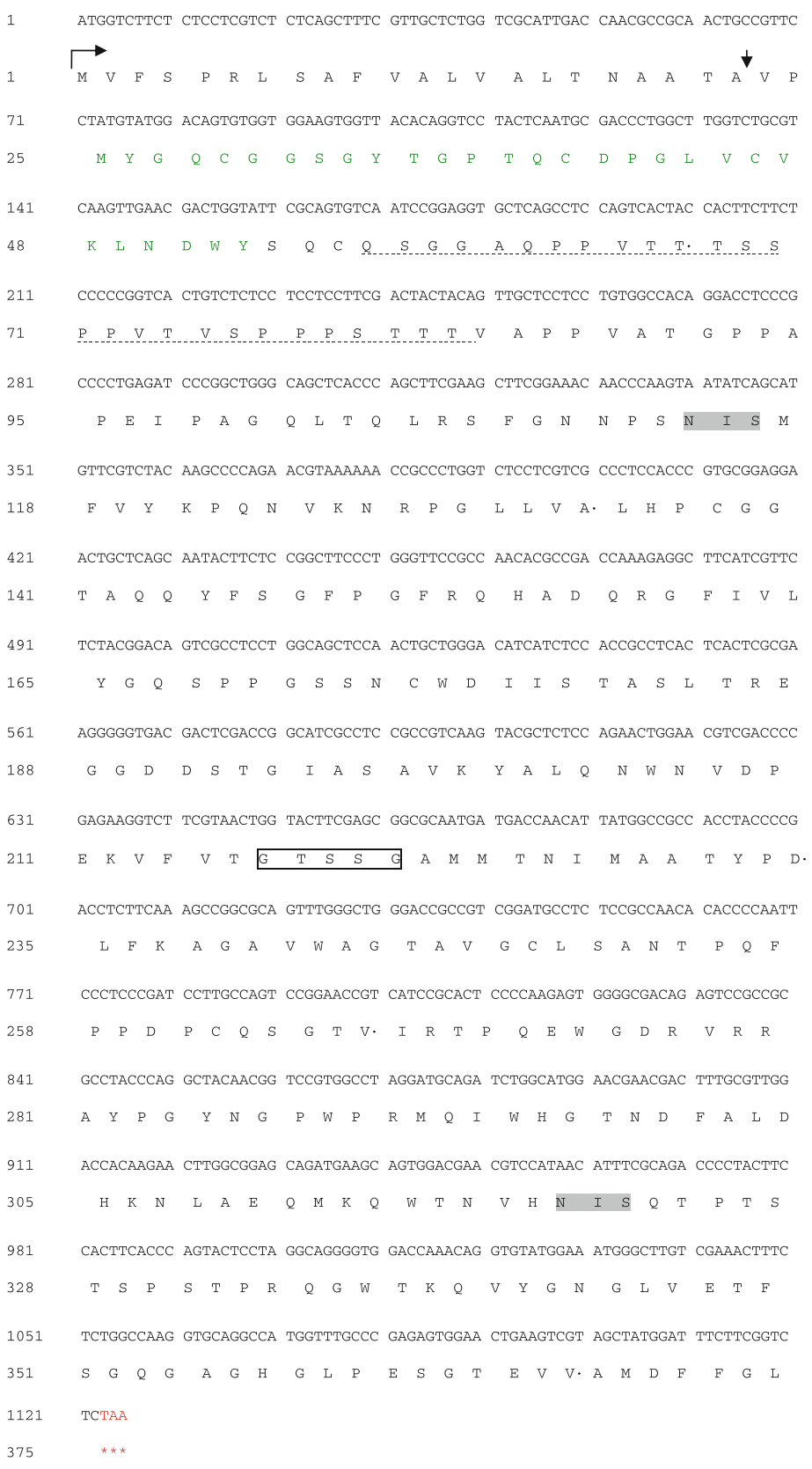

Fig. 1 Gene and deduced amino acid sequences of the AXE from *C. cinerea* Okayama 7 #130. *Black arrow* at the N-terminal region indicates the putative protease cleavage sites inside the potential native signal sequence. Two N-glycosylation sites are highlighted in *grey*. Esterase lipase motif G-X-S-X-G is enclosed in a *box*. Peptide sequence marked in *green* is the CBM1, the peptide sequence *underlined by a dotted line* is the linker region, the remaining sequence is the catalytic domain. (Color figure online)

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1)    15  ....VFAAQNRLQQVTNFGANPNNVGMYVYKPSRVASSPALVVAIHYCSGSAQAYFGGTQ
2)    15  ....ARTTFAQLQQVTNFGPNPTNVGIYAYRPSGLPANPALIVAMHYCTGTAQAYYSGTR
3)    14  ....PVVLAASLTQVNNFGDNPGSLQMYIYVPNKLASKPAIIVAMHPCGGSATEYYGMYD
4)    26  KDVNKRVTAGSLQQVTGFGDNASGTLMYIYVPKNLATNPGIVVAIHYCTGTAQAYYTGSP
5)    27  AEVVPRAT...FTEVRDYGSNPTGTGMWLYVPRNLAAKPAVVVGLHWCSGSAQAYYQGTH
6)    23  ...TASGAANNLTLVSNFGYNPSNVSMYLYVPTKLAPNPPVFVNPHWCHGTAQAAFSGTQ
7)    91  GPPAPEIPAGQLTQLRSFGNNPSNISMFVYKPQNVKNRPGLLVALHPCGGTAQQYFSGFP

1)    71  .YANRADQLGFIVIYPDSPRS.GKCFDVHSPETLKHNAGGDSQGIASMIKYAINNYGVNP
2)    71  .YATLANTYKFIVIYPNAPDS.GGCWDVHTNATLTHDAGGDSLGIASAIRYAINTWGVDR
3)    70  .YHSPADQYGYILIYPSATRD.YNCFDAYSSASLTHNGGSDSLSIVNMVKYVISTYGADS
4)    86  .YAQLAEQYGFIVIYPQSPYS.GTCWDVSSQAALTHNGGGDSNSIANMVTWTISQYNANT
5)    84  .WASNAEKYGYIVIYPQTPYLRSNCWDVSSKKTLMHGGGGVSTSMANMVSFVIAQYGADE
6)    80  .FATLADTYGYIMIFPNSPNAADQCWDVSSTASLSHNGGGDSLAIVNMVKYVLTTYKADP
7)   151 GFRQHADQRGFIVLYGQSPPGSSNCWDIISTASLTREGGDDSTGIASAVKYALQNWNVDP

1)   129 QRVFVTGSSSGAMMTNVMAGSYPDLITATSSYNGVPFGCFAGSG.....EWNSQCATGQI
2)   129 NRVFATGTSSGAMMTNVLAGAYPDLIKAGAAFAGVPYGCFAGPG.....MWNSQCAQGQL
3)   128 SKVYMTGSSSGAIMTNVLAGAYPDVFAAGSAFSGMPYACLYGAGAADPIMSNQTCSQGQI
4)   144 AKVFVTGSSSGAMMTNVMAATYPELFAAAATVYSGVGAGCFYSSSNQ.ADAWNSSCATGSV
5)   143 SRVFATGTSSGAMMTNVLAATYPDVFAAGIAYAGVPAGCFMSADES.ADYWNGTCSLGQT
6)   139 NRVFSMGTSSGAMMTNVLLGVYPDIFAAGSAWAGVAMGCFAANSSA.VDAWSNDCATGQI
7)   211 EKVFVTGTSSGAMMTNIMAATYPDLFKAGAVWAGTAVGCLSANTPQ...FPPDPCQSGTV

1)   184 IKTAAQWGDIARAAYPGYSGPRPKMMIWDGTTDTVLNYKNYAEMIKQWTNVLGVS.QTPT
2)   184 TKTAQQWGDQVRSGYPGYTGSRPKMQIWHGTRDETLNYNNFDEAIKQWTNIFGYS.TTAT
3)   188 QHTGQQWAAYVHNGYPGYTGQYPRLQMWHGTADNVISYADLGQEISQWTTIMGLS.FTGN
4)   203 ISTPAVWGGIAKNMYSGYSGSRPRMQIYHGSADTTLYPQNYYETCKQWAGVFGYNYDSPQ
5)   202 ILTQQQWADVAEAMYPGYTGPRPKMQIYHGSLDTTLYVQNYYETIKQWTGLFGYS.TTPV
6)   198 IKSGAQWKSIVQAAYPGYTGFRPKMWVLHGTDDTTLYPQNLQEEIKEWTTVLGQS.STPL
7)   268 IRTPQEWGDRVRRAYPGYNGPWPRMQIWHGTNDFALDHKNLAEQMKQWTNVHNIS.QTPT
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Fig. 2 Alignment of *Coprinopsis cinerea* AXE with homologous AXEs from protein database. The sequences are (1) *P. indica* (accession no. CCA73570.1), (2) *V. volvacea (*accession no. DQ888226), (3) Ferulic acid esterase B *P. funiculosum* (accession no. AJ291496), (4) *P. purpurogenum* (accession no. AF529173), (5) *G. graminicola* (accession EFQ27328.1), (6) *B. fuckeliana* (accession no. CCD47523.1), (7) *C. cinerea* (accession no. XM001840041.1). Amino acids which are highly conserved among aligned sequences are *light grey* shaded. Potential amino acid residues involved in catalytic activity are *dark grey* shaded

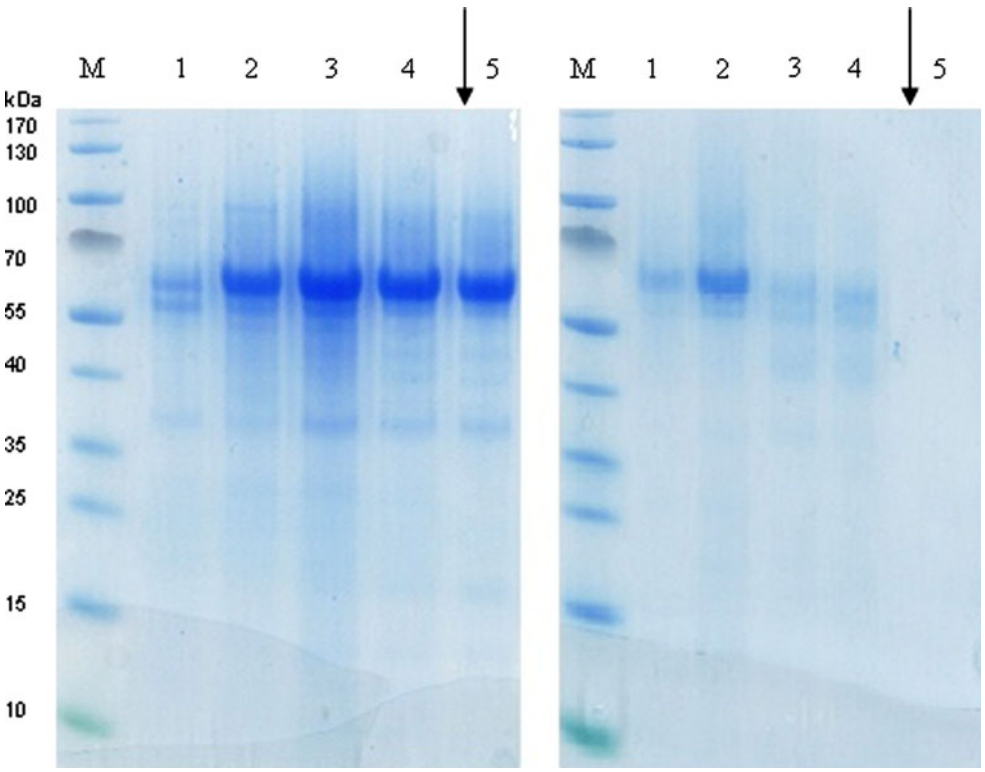

Fig. 3 SDS-PAGE of recombinant AXEs. Gel A: Purified AXE with two additional amino acids at its native N-terminus; Gel B: Purified AXE with its native N-terminus; *Lane* M: Page ruler pre-stained protein ladder (Fermentas, EU); *Lanes* 1–4: Purified his-tagged AXE from days 1 to 4 during induction phase; *Lane* 5: Purified his-tagged AXE on day 7. *Arrow* indicates the last day of induction

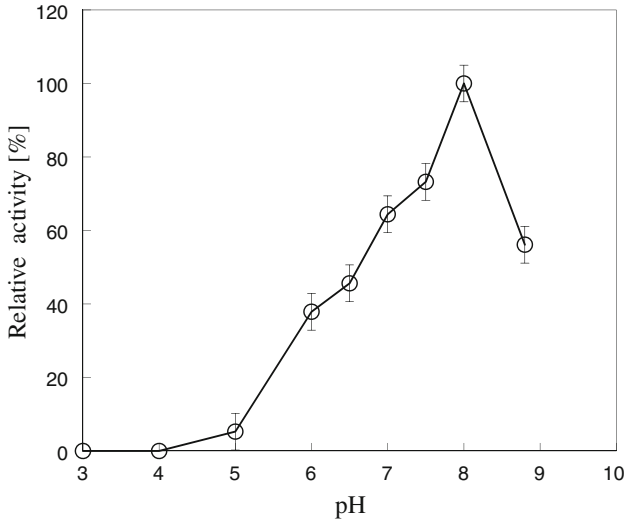

Fig. 4 Effect of pH on recombinant AXE activity. Enzyme was assayed in 100 mM citrate (pH 3–6) and sodium phosphate (pH 7–9) buffers. The highest enzyme activity (99.9 μmol min^{-1} ml^{-1}) was set as 100 %

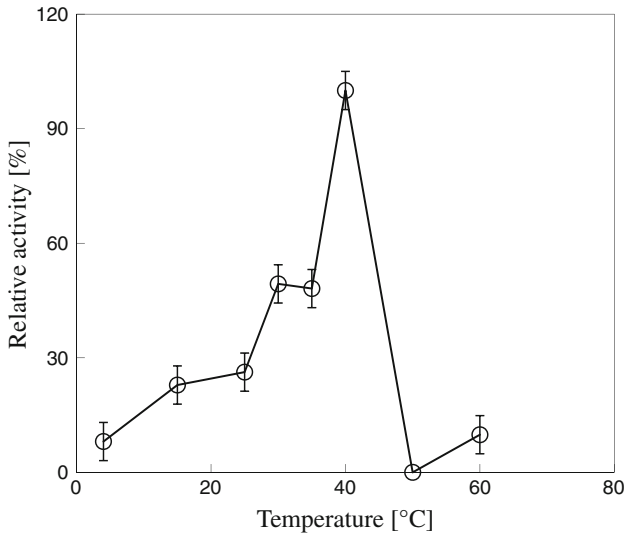

Fig. 5 Effect of temperature on recombinant AXE activity. Enzyme was assayed in 100 mM sodium phosphate buffer at pH8. The highest enzyme activity (99.9 μmol min^{-1} ml^{-1}) was set as 100 %

that N-glycosylation in *P. pastoris* usually leads to a oligosaccharide chain with 8–15 hexose units (Wildt and Gerngross 2005). Glycosylation contributes multiple functions to the protein such as stability, multiplicity and enzyme activity. De-glycosylation of AXEs has been shown to affect their secretory expression, thermal stability and catalytic activity (Tian et al. 2012). The shorter N-glycosylation chain of the recombinant AXE might be favorable it to act with large hemicelluloses substrates leading to more efficient de-acetylation.

The expression levels of both recombinant AXEs with its native N-terminus and with two additional amino acids

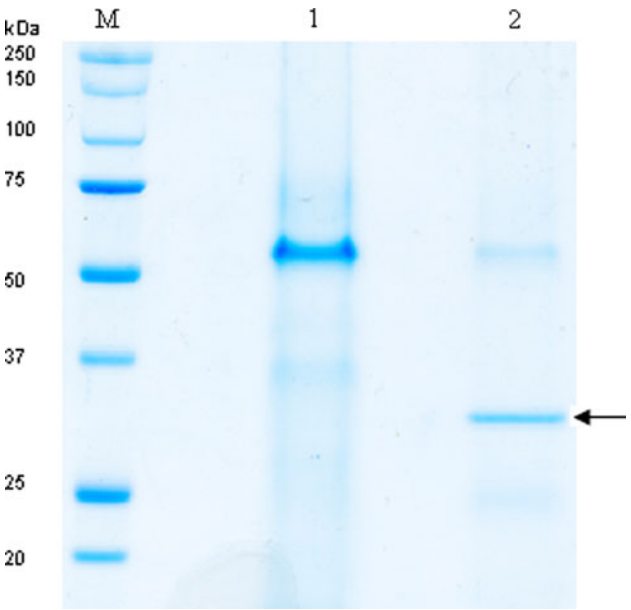

Fig. 6 SDS-PAGE of PNGase F-treated AXE. *Lane* M: Precision plus protein dual color standard (Bio-rad, Germany); *Lane* 1: 10 μg of purified AXE; *Lane* 2: PNGase F-treated AXE. Molecular weight of de-(*N*) glycosylated AXE was 35 kDa

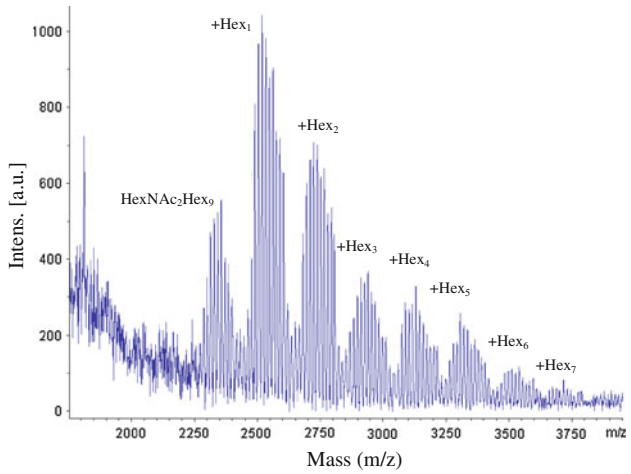

Fig. 7 MALDI-TOF mass spectrum of N-linked glycans from AXE after PNGase F treatment. Positive ions of permethylated N-glycans were detected. N-linked oligosaccharides were identified as $HexNAc_2Hex_{9–16}$. Each *peak* corresponds to one oligosaccharide chain with different permethylation degrees. The mass differences among each group correspond to the mass of one permethylated hexose (204 Da)

at its native N-terminus reached maximum on day 2, indicating that the extra two amino acids in the N terminus did not affect the optimal secretion time of the protein. But the extra two amino acids in the N terminus might help secret more protein judging from the slightly increased expression level in this case. It has been reported that the presence of additional amino acids at the N-terminus of

insulin significantly improved its expression level in *P. pastoris* (Kjeldsen et al. 1999). The same K_m and V_{max} values of the two AXEs with and without the additional two amino acids at its native N-terminus indicates that the enzyme activity is not affected possibly due to no topological change of the enzyme active site by the extra two amino acids. The higher Km for 4-NPA than for 4-NPB indicates a higher affinity of the enzyme to 4-NPB than to 4-NPA, which might be due to the higher hydrophobicity of 4-NPB.

The AXE with its native N-terminus was degraded more rapidly than the AXE with two additional amino acids at its native N-terminus starting from day 3, which might indicate that the two additional amino acids at its native N-terminus helped stabilize the protein making it more resistant to protease cleavages. During the induction phase, proteases were secreted due to the stress responses generated by the change of carbon source from glucose to methanol and the over-expression of the recombinant protein (Sinha et al. 2005). The presence of additional amino acids at the N-terminus might slightly change the configuration of the enzyme, making it difficult for the proteases to access or recognize the cleavage sites. This presumption needs to be further confirmed by comparing the crystal structures of the recombinant proteins.

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