Running title; acetylxylan esterase from *Irpex lacteus*

**Characterization of Acetylxylan Esterase from White-Rot Fungus *Irpex lacteus***

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List of Abbreviations: CE1, carbohydrate esterase family 1; AXE, acetylxylan esterase; FAE, feruloyl esterase; IlAXE1, CE1 acetylxylan esterase from *Irpex lacteus* NK-1; Xylp, xylopyranosyl; GlcA, glucuronic acid; MeGlcA, 4-O-methylglucuronic acid; Araf, arabinofuranosyl; FeA, ferulic acid; CouA, *p*-coumaric acid; *p*NPAc, *p*-nitrophenol acetate; αNAc, α-naphthyl acetate; MUAc, 4-methylumbelliferyl acetate; EtFe, ethyl ferulate; MeCou, methyl *p*-coumarate; CX, corn xylan; DP, degree of polymerization; CBM, carbohydrate binding module
Abstract:

The carbohydrate esterase family 1 (CE1) in CAZy contains acetyl xylan esterases (AXEs) and feruloyl esterases (FAEs). Here we cloned a gene coding for an AXE belonging to CE1 from *Irpex lacteus* (*IIAXE1*). *IIAXE1* was heterologously expressed in *Pichia pastoris*, and the recombinant enzyme was purified and characterized. *IIAXE1* hydrolyzed *p*-nitrophenyl acetate, α-naphthyl acetate and 4-methylumbelliferyl acetate, however, it did not show any activity on ethyl ferulate and methyl *p*-coumarate. We also examined the activity on partially acetylated and feruloylated xylan extracted from corncob by hydrothermal reaction. Similarly, ferulic and *p*-coumaric acids were not liberated, and acetic acid was only detected in the reaction mixture. The results indicated that *IIAXE1* is an acetyl xylan esterase actually reacted to acetyl xylan. However, since *IIAXE1* was unable to completely release acetic acid esterifying xylopyranosyl residues, it is assumed that acetyl groups exhibiting resistance to deacetylation by *IIAXE1* are present in corn cob xylan.

Key words: acetyl xylan esterase, *Irpex lacteus*, xylan, xylooligosaccharide, deacetylation, kinetics
INTRODUCTION

Xylan is the major plant cell wall hemicellulose which shows, depending on plant species or plant organs, large variations in the structure.\(^1\) One of the variations is due to acetylation of Xylp residues. In most cases, Xylp residues of the main xylan chain are esterified with acetic acid at positions 2 or 3 or both positions.\(^2\)\(^3\)\(^4\) In addition to the acetyl groups, plant xylans differ in side chain sugars. In glucuronoxylan or arabinoglucuronoxylan, D-glucuronic acid (GlcA) or 4-O-methyl-D-glucuronic acid (MeGlcA) are linked to the backbone by \(\alpha\)-1,2-glycosidic linkage. In arabinoxylan, \(\alpha\)-L-arabinofuranosyl (Araf) residues are linked to O-2 and or O-3 of xylan backbone. Furthermore, in xylan from annual plants like cereals, the 5-position hydroxyl of Araf residues is frequently esterified by phenolic acids such as ferulic acid (FeA) or \(p\)-coumaric acid (CouA).

Acetylxylan esterases (AXEs; EC 3.1.1.72) are enzymes that liberate acetic acid from acetylated xylan.\(^5\) They are found in carbohydrate esterase (CE) family 1, 4, 5, 6 and 16 (CAZy website available at http://www.cazy.org/).\(^5\)\(^6\) CE1 harbors not only AXEs but also part of feruloyl esterases (FAEs; EC 3.1.1.73) which hydrolyze ester linkages between Araf residues and FeA or CouA in arabinoxylans. To date, several CE1 esterases from filamentous fungi involved in plant cell wall degradation have been purified and characterized. Their activity is usually followed on artificial chromogenic substances such as \(p\)-nitrophenyl acetate (\(p\)NPAc), \(\alpha\)-naphthyl acetate (\(\alpha\)NAc), and 4-methylumbelliferyl acetate (MUAc), ethyl ferulate (EtFe) and methyl \(p\)-coumarate (MeCou). These assays are very convenient, however, they do not provide information on the mode of enzyme action on natural substrate, such as positional specificity of deesterification.

Recently we reported that corn xylan (CX) isolated in high yield from milled corncob by hydrothermal treatment is a mixture of arabinoglucuronoxylan and its oligosaccharides with the degree of polymerization (DP) ranging from 2 to more than 20.\(^7\)\(^8\) This material was found to be partially acetylated and also partially esterified with FeA and CouA at Araf residues\(^9\) which reflects the native corn xylan structure.
Basidiomycete *Irpex lacteus* can rot wood by producing various plant cell wall degrading enzymes\(^{10,11}\), and we have characterized several glycoside hydrolase (GH) family enzymes including 1,4-β-D-endoglucanase (EC 3.2.1.4)\(^{12}\) and 1,4-β-D-cellobiohydrolase (EC 3.2.1.91)\(^{13}\). However, the enzymes belonging to CE family of *I. lacteus* have yet to be characterized. In this paper, we report the cloning and heterologous expression of a cDNA encoding AXE belonging to CE1 from *I. lacteus* NK-1 (*IlAXE1*). In addition, substrate specificity of *IlAXE1* on artificial substrates and extracted CX from corncob is also reported.

**MATERIAL AND METHOD**

**Strain and culture.** A stock culture of *I. lacteus* strain NK-1 was isolated by Nishizawa and Hashimoto\(^{14}\). *Escherichia coli* DH5α, purchased from Takara-Bio Inc. (Shiga, Japan), was used as the host strain for plasmid extraction and was grown at 37°C in Luria-Bertani medium containing 50 μg/mL ampicillin. Plasmid pMD19 (simple) (Takara-Bio) was used as the subcloning vector. The *Pichia pastoris* GS115 strain (Invitrogen, Carlsbad, CA, USA) and plasmid vector pPICZα (Invitrogen) were used for the expression of the AXE.

**Isolation of cDNA coding the acetylxylan esterase *IlAXE1*.** Cells of *I. lacteus* NK-1 were grown in modified Mandel’s medium\(^{15}\) containing microcrystalline cellulose Avicel® PH-101 (Sigma-Aldrich, St. Louis, MO, USA) as the carbon source at 25 °C with orbital shaking (150 rpm) for 6 days. Total RNA was isolated by using TRIZOL® Reagent (ThermoFisher Scientific Inc., Waltham, MA, USA) according to the manufacture’s protocol for filamentous fungi. Total RNA isolated was sent to Takara-Bio company for RNA sequencing (RNA-Seq) and used to synthesize cDNA. For obtaining a full-length cDNA encompassing the whole *IlAXE1* ORF was PCR-amplified from the above first-strand cDNA using two primers: 5’-GCGTACGTTGATCCGCATTTCTTG-3’ (forward primer) and 5’-GAACTACTGCGACATCTATCATACCACC-3’ (reverse primer). Two primers were designed from the result of RNA-Seq analysis. The amplified fragment was subcloned into a pMD19 vector.
and sequenced. The sequence of the cDNA of encoding a full length of \textit{IlAXE1} has been deposited in the DDBJ/EMBL/GenBank database under accession no. BBL86735.1.

\textbf{Expression and purification of recombinant \textit{IlAXE1}.} The cDNA encoding a full length of \textit{IlAXE1} except for the signal peptide was amplified by PCR with PrimeSTAR HS DNA polymerase (Takara-Bio). The PCR reaction employed 300 ng of the total cDNA as a template and 10 pmol of the following primers: 5´-GAATTCCAATCCCAAGTATGGGGTCAG-3´ (forward primer) and 5´-TCTAGAGCGATACCCAAGAACTGGAGG-3´ (reverse primer). (The EcoRI site of the forward primer and the XbaI site of the reverse primer were underlined). The cDNA encoding the mature protein was digested with EcoRI and XbaI and ligated to the corresponding sites of the pPICZ\textalpha{}A vector (Invitrogen).

\textit{Pichia pastoris} GS115 was transformed, and the transformants were selected following the manufacturer’s instructions. The transformants were cultured in 200 mL BMGY medium at 30 °C with shaking (180 rpm) for 1.5 days. The cells harvested, resuspended in 400 mL BMMY medium for 20 h at 30 °C with shaking (180 rpm) with the addition of 0.1 % methanol to maintain induction. The concentration of the secreted recombinant \textit{IlAXE1} reached 240 mg/L at 20 h cultivation. The amount of the enzyme in the medium was estimated from the rate of hydrolysis of \textit{pNPAc}, as described below. \textit{IlAXE1} was purified from the supernatant of the culture using a HisTrap\textsuperscript{TM} HP column (GE Healthcare Biosciences AB, Uppsala, Sweden). After purification, the concentration of the protein was measured by the protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with bovine serum albumin as a standard protein. The purified \textit{IlAXE1} were separated by 12.5% SDS-PAGE and transferred to a PVDF transfer membrane 0.45 \textmu{}m (Amersham Hybond\textsuperscript{TM}P, GE Healthcare). Membranes were blocked in Blocking One buffer (Nacalai tesque, Tokyo, Japan) and probed with the primary mouse anti-His-tag antibody (MBL Co., Ltd., Aichi, Japan), followed by incubation with an AP-conjugated anti-mouse IgG secondary antibody (Promega, Madison, WI, USA).
**Enzyme activity assays.** A solution of 0.5 mM of substrate in 0.1 M potassium phosphate buffer (pH 6.0) (200 μL), 0.2 μg of the purified IlAXE1 was added and incubated at 30 °C in the 96-well microplate. The changes in the absorption spectra of the hydrolysis products of each substrate were monitored in suitable wavelength range by using the multiskan GO spectrophotometer (ThermoFisher Scientific) at 30 sec intervals during 2 min. The released p-nitrophenol, α-naphtol, and 4-methylumberiferone were quantified from its standard curve monitored absorbance at 400 nm, 322 nm, and 338 nm, respectively.

Kinetic data of IlAXE1 for the acetyl esters were obtained as described above by incubating 0.2 μg of the enzyme with the following concentration ranges of the substrates: MUAc 0.005–1.000 mM, pNPAc 0.050–1.500 mM and αNAc 0.050–0.500 mM. The values of \( V_{\text{max}} \) and \( K_m \) were calculated by a non-linear least squares optimization of the Michaelis-Menten equation. Since the \( K_m \) value for αNA could not be obtained due to the low solubility of αNA above 1.0 mM, its \( k_{\text{cat}}/K_m \) value was determined from the slope of \( s-v \) plots at the concentrations from 0.050 to 0.500 mM.

**Activity on acetylated xylan.** A solution of 1.0 % (w/v) CX in 50 mM phosphate buffer (pH 6.0) was incubated with 6.5 μg of IlAXE1 at 30 °C. Aliquots (0.5 mL) were withdrawn from each reaction mixture every 10 min during 60 min, and the reaction was terminated by adding 100 μL of 1.0 M HCl. Heat-denatured enzyme was used in control reaction mixtures. The released acetic acid was analyzed by HPLC connected to cation-exchange column RSpak KC-811 (Showa Denko K.K., Tokyo, Japan) with a pH indicator as a post-column regent, and the released FeA or CouA was analyzed by HPLC connected to Shim-pack XR-ODS column (Shimadzu Corporation, Kyoto, Japan) as previously described. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of acetic acid per min from the substrate. The content of acetyl group in CX was estimated by measuring the amount of acetic acid released after alkaline hydrolysis (70°C, 3 h) of 1.0 % CX with 1 M NaOH (final concentrations).
RESULTS AND DISCUSSION

Isolation of a cDNA coding for AXE.

The 1,098 bp of cDNA fragment with ATG start codon and TAA stop codon was amplified by PCR. The deduced amino acid sequence corresponds to 365 amino acid residues including the secretion signal peptide (Met1 to Gly20) predicted using the SignalP v4.1 server (http://www.cbs.dtu.dk/services/SignalP/). In addition, the sequence has suggested that the primary structure of this protein contains two domains, the catalytic core domain homologous to a CE1 esterase and the family 1 carbohydrate binding module (CBM1) at the N-terminal region (Gly26 to Ile56). The genome sequence of *I. lacteus* F17 (GenBank accession number: MQVO00000000) has been reported by Yao et al. in 2017.11) One homologous gene was found in the whole genome of *I. lacteus* F17, and the homology of both deduced amino acid sequences was 95 % identity. Furthermore, a protein BLAST program (http://www.ncbi.nlm.nih.gov/blast) revealed that the sequence of the catalytic core domain (Phe110 to Ala365) showed high identity with sequences of several characterized AXEs; the highest with *PcAXE2* from *Phanerochaete chrysosporium* (77 %, AEX99761.1),18) *VvAXE* from *Volvariella volvacea* (56 %, ABI63599)19) and *MtAXE3* from *Chrysosporium lucknowense* C1 (47 %, ADZ98864).20) The three amino acid residues, Ser209, Asp292 and His349, which are thought to be the catalytic triad in the *Irplex lacteus* CE1 esterase are also conserved in the above mentioned AXEs. Based on this information, we named this protein as *IlAXE1*.

Expression and general properties of *IlAXE1*.

Although the molecular mass calculated from the primary structure of expressed *IlAXE1* is 38.4 kDa, the purified *IlAXE1* was shown as a single band of approximately 60 kDa (Fig. 1). *IlAXE1* contains two sites for *N*-glycosylation (Asn320 and Asn332) and many sites for *O*-glycosylation located at the linker region between CBM1 and the catalytic domain. Although the position of the
band shifted to 42 kDa after deglycosylation by N-glycosidase F, it was still larger than that of theoretical molecular mass indicating that the recombinant *IlAXE1* remained still partially glycosylated.

In order to evaluate the esterase activity, the change in absorption spectra of ester substrates were measured during incubation with purified *IlAXE1*. The absorption spectra showed that *IlAXE1* hydrolyzed three acetate esters (pNPAc, αNAc, and MUAc), whereas no activity for EtFe and MeCou was observed (Fig. 2). This result is very similar to those reported for other fungal AXEs. To evaluate the velocity of hydrolysis reaction to pNPAc, αNAc, and MUAc, the calibration curves were prepared for *p*-nitrophenol at 400 nm, α-naphthol at 322 nm and 4-methylumbelliferone at 338 nm as described previously. The specific activities of *IlAXE1* for various substrates are listed in Table 1. *IlAXE1* showed the highest activity for MUAc (27.0 ± 0.9 U/mg), which is 2.3- and 4.6-times higher than those of pNPAc (11.8 ± 0.8 U/mg) and αNAc (5.9 ± 0.4 U/mg), respectively. Similar specific activities for these substrates were reported for *VvAXE1*. In contrast to *IlAXE1*, an extracellular carbohydrate esterase from *Coprinopsis cinerea* (*CcEst1*) showed both AXE and FAE activities and the highest activity toward pNPAc. However, most of AXEs belonging to CE1 family show only AXE activity, and *IlAXE1* appears to belong to this group of CE1 esterases.

Table 2 presents the determined kinetics parameters and Fig. 3 shows the relationship between the velocity and substrate concentrations, and the calculated Michaelis-Menten equation curves based on three independent experiments. The *k*<sub>cat</sub> values of *IlAXE1* for MUAc and pNPAc were almost the same, but the *K*<sub>m</sub> value for MUAc was one fourth of that for pNPAc. When the final concentration of αNAc in the reaction mixture exceeded 0.5 mM, the substrate became insoluble and inhibition of the hydrolytic reaction was observed (Fig. 3C).

Among acetate esters, MtFe and MeCou, *IlAXE1* can hydrolyze only the ester linkage of acetate esters and liberate acetic acid. This means that *IlAXE1* recognizes the acetyl group of ester substrates.
On the other hand, the differences in $K_m$ values of IIAXE1 for acetate esters (MUAc and $p$NPAc) indicated that recognition of chromophore part of ester substrates is also important for the enzyme activity.

**AXE activity on acetyl xylan isolated by hydrothermal reaction.**

Partially acetylated and feruloylated CX isolated from milled corn cobs in a continuous hydrothermal reactor under reported conditions (190 °C, 1.8 MPa, 13 min) was used as a natural substrate to examine the ability of IIAXE1 to deesterify acetylated Xylp residues and liberate ferulic acid from Araf residues. The composition of this material with DP ranging of 2 – 20 was reported in our previous work: glucose, 12.1 % (w/v), xylose 53.5 % (w/v), arabinose 2.3 % (w/v), glucuronic acid 3.6 % (w/v), acetic acid 3.9 % (w/v), ferulic acid, or $p$-coumaric acid 1.8 % (w/v). As shown in Fig. 4, free FeA and CouA together with acetic acid, were detected only after alkali treatment of CX. IIAXE1 released acetic acid but no FeA and CouA. This result was consistent with behavior of IIAXE1 on synthetic chromogenic substrates and confirmed the lack of feruloyl esterase activity of this enzyme. The specific activity of IIAXE1 (16.8 ± 0.2 U/mg) for CX was similar to the specific activity on the acetyl-4-$O$-methylglucuronoxylan of other AXEs, like OsAcXE from Oripinomyces sp. (CE6, 22.8 ± 3 U/mg), and MtAcE from Myceliophthora thermophila (CE16, 20.1 ± 2 U/mg), whereas it was 3.1 times higher than that of AnAcXE from Aspergillus nidulans (CE1, 5.4 ± 0.7 U/mg). After 24 h reaction, IIAXE1 removed 76.9 % of total acetyl groups present in CX. This result means that IIAXE1 did not remove acetyl groups from the xylan chain completely, similarly to results reported in other AXEs. Recently, we have also reported that 3-$O$-positioned acetyl group of Xylp residues 2-$O$-substituted by MeGlcA or feruloylated Araf residues have not been deesterified after treatment by AXE belonging to CE6. These modification patterns of Xylp with acetyl group and other substitutions were experimentally detected in CX used in this study, which was prepared from corn cob by hydrothermal treatment.
patterns affect reactivity of IlAXE1 to acetyl xylan. In future, it is needed to clarify the relationships between deacetylation activity of IlAXE1 and modification patterns of acetyl groups for understanding of enzymatic degradation of native xylan.

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REFERENCES

1) H.V. Scheller and P. Ulvskov: Hemicelluloses. *Annu. Rev. Plant Biol.*, **61**, 263–289 (2010).

2) H.O. Bouveng, P.J. Gregg, and B. Lindberg: Position of O-acetyl groups in birch xylan. *Acta. Chem. Scand.*, **14**, 742–748 (1960).

3) A. Teleman, J. Lundquist, F. Tkerneld, H. Stalbrand, and O. Dahlman: Characterization of acetylated 4-O-methylglucuronoxylan isolated from aspen employing $^1$H and $^{13}$C NMR spectroscopy. *Carbohydr. Res.*, **329**, 807–815 (2000).

4) D.V. Evtuguin, J.L. Tomas, A.M.S Silva, and C.P. Neto: Characterization of an acetylated heteroxylan from *Eukalyptus globulus* Labill. *Carbohydr. Res.*, **338**, 597–604 (2003).

5) P. Biely: Microbial carbohydrate esterases deacetylating plant polysaccharides. *Biotechnol. Adv.*, **30**, 1575–1588 (2012).

6) V. Lomberd, H.G. Ramulu, E. Drula, P.M. Coutinho, and B. Henrissat: The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic. Acids. Res.*, **42**, 490–495 (2014).

7) S. Makishima, M. Mizuno, N. Sato, K. Shinji, M. Suzuki, K. Nozaki, F. Takahashi, T. Kanda, and Y. Amano: Development of continuous flow type hydrothermal reactor for hemicellulose
fraction recovery from corn cob. Bioresour. Technol., 100, 2842–2848 (2009).

8) N. Sato, K. Shinji, M. Masahiro, K. Nozaki, M. Suzuki, S. Makishima, M. Shiroishi, T. Onoda, F. Takahashi, T. Kanda, and Y. Amano: Improvement in the productivity of xylooligosaccharides from waste medium after mushroom cultivation by hydrothermal treatment with suitable pretreatment. Bioresour. Technol., 101, 6006–6011 (2010).

9) N. Sato, Y. Takano, M. Masahiro, K. Nozaki, S. Umemura, T. Matsuzawa, Y. Amano, and S. Makishima: Production of feruloylated arabino-oligosaccharides (FA-AOs) from beet fiber by hydrothermal treatment. J. Supercrit. Fluids, 79, 84–91 (2013).

10) D. Salvachúa, A.T. Martínez, M. Tien, M.F.L. Lucendo, F. García, V.I. Ríos, M.J. Martínez, and A. Prieto: Differential proteomic analysis of the secretome of Iepex lacteus and other white-rot fungi during wheat straw pretreatment. Biotechnol. Biofuels, 6, (2013).

11) M. Yao, W. Li, Z. Duan, Y. Zhang, and R. Jia: Genome sequence of the white-rot fungus Irpex lacteus F17, a type strain of lignin degrader fungus. Stand. Genomic. Sci., 12, (2017).

12) H. Toda, S. Takada, M. Oda, Y. Amano, T. Kanda, M. Okazaki, and M. Shimosaka: Gene cloning of an endoglucanase from the basidiomycete Irpex lacteus and its cDNA expression in Saccharomyces cerevisiae. Biosci. Biotechnol. Biochem., 69, 1262-1269 (2005).

13) H. Toda, N. Nagahama, Y. Amano, K. Nozaki, T. Kanda, M. Okazaki, and M. Shimosaka: Gene cloning of cellobiohydrolase II from the white rot fungus Irpex lacteus MC-2 and its expression in Pichia pastoris. Biosci. Biotechnol. Biochem., 72, 3142-3247 (2008).

14) K. Nishizawa and Y. Hashimoto: Cellulose-splitting enzymes. VI. Difference in the specificities of cellulase and β-glucosidase from Irpex lacteus. Arch. Biochem. Biophys., 81, 211-222 (1959).

15) M. Mandels and J. Weber: The production of cellulases. Adv. Chem. Ser., 95, 391-414 (1969).

16) A. Wada, M. Bonoshita, Y. Tanaka, and K. Hibi: A study of a reaction system for organic acid analysis using a pH indicator as post-column reagent. J. Chromatogr., 291, 111–118 (1984).

17) K. Glowniak, G. Zgórką, and M. Kozyra: Solid-phase extraction and reversed-phase high-
performance liquid chromatography of free phenolic acids in some *Echinacea* species. *J. Chromatogr. A*, 730, 25–29 (1996).

18) N.D. Huy, S. Thiyagarajan, D. Kim, and S. Park: Cloning and characterization of a novel bifunctional acetyl xylan esterase with carbohydrate binding module from *Phanerochaete chrysosporium*. *J. Biosci. Bioeng.*, 115, 507–513 (2013).

19) S. Ding, J. Cao, R. Zhou, and F. Zheng: Molecular cloning, and characterization of a modular acetyl xylan esterase from the edible straw mushroom *Volcariella volvacea*. *FEMS Microbiol. Lett.*, 274, 204–310 (2007).

20) L. Pouvreau, M.C. Jonathan, M.A. Kabel, S.W. Hinz, H. Gruppen, and H.A. Schols: Characterization and mode of action of two acetyl xylan esterases from *Chrysosporium lucknowense* C1 active towards acetylated xylans. *Enzyme Microb. Technol.*, 49, 312–320 (2011).

21) T. Koseki, S. Furuse, K. Iwano, H. Sakai, and H. Matsuzawa: Biochemical characterization of recombinant acetyl xylan esterase from *Aspergillus awamori* expressed in *Pichia pastoris*: Mutational analysis of catalytic residues. *Biochem. J.*, 1479, 7–13 (2005).

22) D. Komiya, A. Hori, T. Ishida, K. Igarashi, M. Samejima, T. Koseki, and S. Fushinobu: Crystal structure and substrate specificity modification of acetyl xylan esterase from *Aspergillus luchuensis*. *Appl. Microbiol. Biotechnol.*, 83, e01251–17 (2017).

23) T. Koseki, Y. Miwa, T. Akao, O. Akita, and K. Hashizume: An *Aspergillus oryzae* acetyl xylan esterase: Molecular cloning and characteristics of recombinant enzyme expressed in *Pichia pastoris*. *J. Biotechnol.*, 121, 381–389 (2006).

24) F.J.M. Kormelink, B. Lefebvre, F. Strozyk, and A.G.J. Voragen: Purification and characterization of an acetyl xylan esterase from *Aspergillus niger*. *J. Biotechnol.*, 27, 267–282 (1993).

25) H. Chung, S. Park, H. Kim, M. Yang, and D. Kim: Cloning the gene encoding acetyl xylan esterase from *Aspergillus ficuum* and its expression in *Pichia pastoris*. *Enzyme Microb. Technol.*, 31, 384–391 (2002).
26) C. Pai, Z. Wu, M. Chen, Y. Zeng, J. Chen, C. Duan, M. Li, and J. Liu: Molecular cloning and characterization of a bifunctional xylanolytic enzyme from *Neocallimastix patriciarum*. *Appl. Microbiol. Biotechnol.*, **85**, 1451–1462 (2010).

27) K. Hashimoto, S. Kaneko, and M. Yoshida: Extracellular carbohydrate esterase from the basidiomycete *Coprinopsis cinerea* released ferulic and acetic acid from xylan. *Biosci. Biotechnol. Biochem.*, **74**, 1722–1724 (2010).

28) M. Qi, P. Wang, L.B. Selinger, L.J. Yanke, R.J. Forster, and T.A. McAlister: Isolation and characterization of a ferulic acid esterase (Fae1A) from the rumen fungus *Anaeromyces mucronatus*. *J. Appl. Microbiol.*, **110**, 1341–1350 (2011).

29) V.F. Crepin, C.B. Faulds, and F. Connerton: A non-modular type B feruloyl esterase from *Neurospora crassa* exhibits concentration-dependent substrate inhibition. *Biochem. J.*, **370**, 417–427 (2003).

30) G. Mai-Gisondi, and E.R. Master: Colorimetric detection of acetyl xylan esterase activities. *Methods Mol. Biol.*, **1588**, 45–47 (2017).

31) G. Mai-Gisondi, H. Maaheimo, S. Chong, S. Hinz, M. Tenkanen, and E. Master: Functional comparison of versatile carbohydrate esterases from families CE1, CE6, and CE16 on acetyl-4-0-methylglucuronoxylan and acetyl-galactomannan. *Biochem. Biophys. Acta*, **1861**, 2398–2405 (2017).

32) K.G. Neumüller, A.C. de Souza, J.H. van Rijin, H. Streekstra, H. Gruppen, and H.A Schols: Positional preferences of acetyl esterases from different CE families towards acetylated 4-O-methyl glucuronic acid-substituted xylo-oligosaccharides. *J. Biotechnol.*, **233**, 228–236 (2016).

33) I. Uhliariková, M. Vršanská, B.V. McCleary, and P. Biely: Positional specificity of acetylxyylan esterases on natural polysaccharide: An NMR study. *Biochem. Biophys. Acta*, **1830**, 3365–3372 (2013).

34) T. Arai, P. Biely, I. Uhliariková, N. Sato, S. Makishima, M. Mizuno, K. Nozaki, S. Kaneko, and
Y. Amano: Structural characterization of hemicellulose from corn cob in continuous flow type hydrothermal reactor. *J. Biosci. Bioeng.*, **127**, 222–230 (2019).
Table 1. Substrate specificity of *IlAXE1* and a comparison with other characterized esterases belonging to CE1 family.

| Name       | Organism          | Specific activity [U/mg] | Ref.         |
|------------|-------------------|-------------------------|--------------|
|            |                   | Acetate ester | Ferulate ester | *p*-Coumarate ester |
|            |                   | *p*NPac | αNAc | MUAc | EtFe (or MtFe) | MeCou |
| *IlAXE1*   | *Irpex lacteus*   | 11.8±0.9 | 5.9±0.4 | 27.0±0.9 | ND | ND | This study |
| *PcAXE2*   | *Phanerochaete chrysosporium* | 39.86 | - | - | - | - | 18 |
| *VvAXE1*   | *Volvariella volvacea* | ND | 961 | 24733 | - | - | 19 |
| *AIAXEA*   | *Aspergillus luchuensis* | 21.9±1.7 | - | - | (ND) | ND | 21, 22 |
| *AoAXEA*   | *Aspergillus oryzae* | 24.8±3.4 | - | - | - | - | 23 |
| *AnAcXE*   | *Aspergillus niger* | 32.3 | - | - | - | - | 24 |
| *AfAXE*    | *Aspergillus ficuum* | 32.5 | - | - | - | - | 25 |
| *AxeS20E*  | *Neocallimastix patriciarum* | ND | ND | 580.3±62.5 | - | - | 26 |
| *CcEst1*   | *Coprinopsis cinereal* | 66.2 | 2.67 | - | (1.26) | 0.78 | 27 |
| *AlFAEA*   | *Aspergillus luchuensis* | - | 1.3 | - | (9.01) | 0.11 | 22 |
| *AmCE1*    | *Anaeromyces mucronatus* | 3.21±0.17 | 1.77±0.11 | - | 7.75±0.24 | 12.51±0.46 | 28 |
| *NcFae-1*  | *Neurospora crassa* | - | 6.65 | - | (8.97) | 20.87 | 29 |

The top line background showed that the value of U/mg protein (mean ± SD., determined by three independent experiments) obtained in this report. Specific activity was determined by using *p*NPac, αNA, MUAc for checking acetic acid releasing activity and EtFe, MeCou for checking ferulic acid and *p*-coumaric acid releasing activity. Other data are derived from previous papers, *a* U/μmol as used as units of specific activity, and *b* methyl ferulate (MtFe) as used as a substrate of ferulic ester. ND, no detectable activity. —, undetermined.
Table 2. Kinetic parameters of IIAXE1.

| Substrate                  | Kinetic parameters |   |   |   |
|----------------------------|--------------------|---|---|---|
|                            | $K_m$ (mM) | $V_{max}$ (mM·min$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (mM$^{-1}$·s$^{-1}$) |
| 4-Methylumbelliferyl acetate | MUAc    | 0.4 ± 0.1 | 0.057 ± 0.010 | 33.8 ± 5.7 | 85.5 |
| $p$-Nitrophenyl acetate    | pNPAc   | 1.4 ± 0.3 | 0.049 ± 0.007 | 29.1 ± 4.3 | 20.6 |
| $\alpha$-Naphtyl acetate  | $\alpha$NAc | N.D.   | N.D.   | N.D.   | 12.1 ± 8.1 |

$K_m$ and $k_{cat}$ values are shown with standard deviations from three independent experiments. N.D.: values could not be obtained due to the low solubility of $\alpha$NA at the concentration above 1.0 mM.
**Fig. 1.** SDS-PAGE and western blot analysis of purified recombinant *IIAXE1*.

Proteins in the gel were stained with silver staining kit (EzStain Silver, ATTA, Tokyo, Japan) Lane M, molecular mass markers (EzStandard, ATTA, Tokyo, Japan); lane 1, purified *IIAXE1*; lane 2, *N*-glycosidase F treated *IIAXE1*; lane 3, Endo-H treated *IIAXE1*; lane WB, western blot analysis of purified *IIAXE1*.

**Fig. 2.** Changes in absorption of five chromogenic substrates during incubation with *IIAXE1*.

*IIAXE1* was incubated in 0.1 M potassium phosphate buffer at pH6.0 with 0.5 mM of *p*-nitrophenyl acetate (*p*NPAc) (A); *α*-naphthyl acetate (*α*NA) (B); 4-methylumbelliferyl acetate (MUAc) (C); ethyl ferulate (EtFe) (D); methyl *p*-coumarate (MeCou) (E). The arrows show a peak at a specific wavenumber which used for calculating the esterase activity.

**Fig. 3.** Kinetics of hydrolysis of artificial substrates by *IIAXE1*

Kinetic data of *IIAXE1* were obtained with the following concentration ranges of the substrates: (A) *p*NPAc 0.050 - 1.500 mM; (B) MUAc 0.005 - 1.000 mM; (C) *α*NAc 0.050 - 0.500 mM. Experimental data points are shown as circles. The best fits obtained by a non-linear least squares optimization of the Michaelis-Menten equation are shown as dashed lines.

**Fig. 4.** HPLC analysis of reaction products released from CX by *IIAXE1*.

HPLC chromatograms showed released (A) acetic acid (B) *p*-coumaric acid or ferulic acid. Comparison of enzyme reaction products released from CX by 6.5 μg of *IIAXE1* from reaction with 1.0% substrate. The control reaction was used by heat-denatured enzyme (Boiling, 10 min). The chromatogram showed a standard peak of releasing products (dot line, A, 0.1 mM of acetic acid; B 0.01 mM of *p*-coumaric acid and 0.01 mM of ferulic acid) and 1 M NaOH treated CX (70 °C, 3 h) as it appeared before enzyme hydrolysis (black line, Alkaline treatment, from 1.0% of substrate).
Fig. 1
Fig. 2
Fig. 3
Fig. 4