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Identification and Characterization of Novel Intracellular α-Xylosidase in *Aspergillus oryzae*

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Abstract: α-Xylosidase releases xylopyranosyl side chains from xyloglucan oligosaccharides and is vital for xyloglucan degradation. Previously, we identified and characterized two α-xylosidases, intracellular AxyA and extracellular AxyB, in *Aspergillus oryzae*. In this study, we identified a third α-xylosidase, termed AxyC, in *A. oryzae*. These three *A. oryzae* α-xylosidases belong to the glycoside hydrolase family 31, but there are clear differences in substrate specificity. Both AxyA and AxyB showed much higher hydrolytic activity toward isoprimeverose (α-D-xylopyranosyl-1,6-glucose) than *p*-nitrophenyl α-D-xylopyranoside. In contrast, the specific activity of AxyC toward the *p*-nitrophenyl substrate was approximately 950-fold higher than that toward isoprimeverose. Our study revealed that there are multiple α-xylosidases with different substrate specificities in *A. oryzae*.

Key words: *Aspergillus oryzae*, α-xylosidase, glycoside hydrolase family 31, xyloglucan

INTRODUCTION

Xyloglucan is a major hemicellulosic polysaccharide found in land plant cell walls and seeds and plays an important role as a matrix polysaccharide in cell walls[1)2)] and a storage polysaccharide in seeds.[3)] Xyloglucan has β-1,4-glucan as its main chain, and xylopyranosyl side chains are attached at the C6 position of the glucopyranosyl residues. In addition, other saccharides such as galactose, arabinose, and fucose are attached to the side chains of xyloglucan.[4)5)] The structure of the side chains of xyloglucan are presented by one-letter codes, that is, X: an α-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl segment, L: a β-D-galactopyranosyl-(1→2)-α-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl segment, and G: an unbranched glucopyranosyl residue.[6)7)] Microorganisms produce several types of glycosidases that degrade and assimilate xyloglucan.

Aspergillus oryzae has been used to produce traditional Japanese fermented foods such as miso, soy sauce, and Japanese rice wine (sake). *A. oryzae* produces many polysaccharide degradation-related enzymes[8)9)] including xyloglucan degradation-related enzymes such as two xyloglucan-specific endo-β-1,4-glucanases (xyloglucanases, Xeg5A and Xeg12A, EC 3.2.1.151),[10)] isoprimeverose-producing oligoxyloglucan hydrolase (IpeA, EC 3.2.1.120),[11)12)] β-galactosidase (LacA, EC 3.2.1.23),[13)] and two α-xylosidases (AxyA and AxyB, EC 3.2.1.177).[14)15)] Xeg5A and Xeg12A degrade xyloglucans into xyloglucan oligosaccharides.[10)] Subsequently, xyloglucan oligosaccharides are degraded by the cooperative action of IpeA and LacA. IpeA releases isoprimeverose [α-D-xylopyranosyl-(1→6)-D-glucose] from the non-reducing ends of xyloglucan oligosaccharides,[16)17)] whereas LacA releases galactose from the side chains of xyloglucan oligosaccharides.[13)] Isoprimeverose is then hydrolyzed to D-xylose and D-glucose by AxyA and AxyB.[14)15)] AxyB contains an N-terminal signal sequence for its secretion. In contrast, AxyA does not have an N-terminal signal sequence for secretion, suggesting that AxyA and AxyB are intracellular and extracellular α-xylosidases, respectively. In addition to these glycosidases, lytic polysaccharide monooxygenases are involved in xyloglucan degradation in *A. oryzae*.[18)]

α-Xylosidases have been identified in many microorganisms and plants.[19)20)21)22)] Based on these amino acid sequences, α-xylosidases belong to the glycoside hydrolase family 31 (GH31).[23)] GH31 is a large family that is divided into 20 subfamilies.[24)] In addition to α-xylosidases, GH31 includes α-glucosidases (EC 3.2.1.20), α-galactosidases (EC 3.2.1.22), α-mannosidases (EC 3.2.1.24), isomaltosyltransferases (EC 2.4.1.387), and other enzymes.[23)]

While many α-xylosidases prefer isoprimeverose as a substrate,[19)] some enzymes prefer much larger xyloglucan oligosaccharides, such as XXXG (Glc_4Xyl_3) and XLLG ($Glc_4Xyl_3Gal_2$), than isoprimeverose.[25)] Both *A. oryzae* AxyA and AxyB prefer isoprimeverose as a substrate over xyloglucan oligosaccharides, such as XXXG and XLLG, and their hydrolytic activities toward *p*NP α-D-xylopyranoside are quite low.[14)15)] In the current study, we identified and characterized a third α-xylosidase, AxyC, in *A. oryzae*. The substrate specificity and amino acid sequence of AxyC are

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markedly different from those of AxyA and AxyB.

MATERIALS AND METHODS

Materials. Xyloglucan was purchased from Megazyme (Wicklow, Ireland). Isoprimeverose and xyloglucan oligosaccharides were prepared as previously described.[12] *p*-Nitrophenyl (*p*NP) substrates were purchased and prepared as described previously.[26]

Cloning, heterologous expression, and purification of AxyC. Polymerase chain reaction (PCR) was used to amplify *axyC* gene using the following primers: 5′-AACTATTTCGA AACGATGCTCTATGCCGAAGACGATAAGC-3′(Primer 1 shown in Fig. 1) and 5′-ATGATGATGGTCGACCGC CTTGACGAAAACAGGCATTG-3′ (Primer 4 shown in Fig. 1), and cDNA of *A. oryzae* as a template. cDNA was synthesized from the total RNA extracted from *A. oryzae* RIB40 cells cultured in xylose medium, as described previously.[12] A pGAPZ vector (Invitrogen, Waltham, MA, USA) was linearized by PCR using the following primers: 5′-GTCGACCATCATCATCATCATCATTGAG-3′ and 5′-CGTTTCGAAATAGTTGTTCAATTGATTG-3′. These two amplified DNA fragments were connected using an In-Fusion HD Cloning Kit (Takara Bio Inc., Shiga, Japan) to obtain the pGAPZ-AxyC-His_6 plasmid. The pGAPZ-AxyC-His_6 plasmid was linearized by PCR using the following primers: 5′-AGGAAATTTTACTCTGCTGGAGA GCTTC-3′, and 5′-AGGGACGGTAACGGGCGGTGG-3′, and the amplified DNA fragment was introduced into the methylotrophic yeast *Pichia pastoris* X-33. The *P. pastoris* cells harboring pGAPZ-AxyC-His_6 were cultured in YPD (1 % yeast extract, 2 % peptone, and 2 % glucose) medium containing 100 mM potassium phosphate buffer (pH 6.0) at 30 ˚C on a shaker at 150 rpm for 24 h. After cultivation, *P. pastoris* cells were collected by centrifugation (6,000 × *g*, 3 min) and resuspended in 20 mM sodium phosphate buffer (pH 7.4) containing 300 mM NaCl, 20 mM imidazole, and a cOmplete EDTA-free Protease Inhibitor Cocktail (F. Hoffmann-La Roche AG, Basel, Switzerland). Resuspended *P. pastoris* cells were disrupted using 1 mm glass beads and a vortex mixer. Cell debris was removed by centrifugation (10,000 × *g*, 10 min, 4 ˚C) and filtration (0.22 μm), and His_6-tagged-AxyC was purified using Ni^{2+} affinity column packing His60 Ni Superflow Resign (Clontech Laboratories Inc., Mountain View, CA, USA). The crude cell extract was loaded onto a Ni^{2+} affinity column, and the column was washed with 20 mM sodium phosphate buffer (pH 7.4) containing 300 mM NaCl and 20 mM imidazole. AxyC-His_6 was eluted using 20 mM sodium phosphate buffer (pH 7.4) containing 300 mM NaCl and 500 mM imidazole. Purified AxyC was concentrated and desalted using a Vivaspin Turbo 15–30K (Sartorius, Göttingen, Germany) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by measuring UV the absorbance at 280 nm using a NanoDrop Lite (Thermo Fisher Scientific, Waltham, MA, USA). The extinction coefficient for His_6-tagged-AxyC (180,055 M^{-1} cm^{-1}) was calculated based on the amino acid sequence using ProtParam (http://web.expasy.org/portparam/).

AxyC was subjected to gel filtration chromatography (Superdex 200 increase 10/300 GL; Cytiva, Tokyo, Japan) equilibrated with phosphate buffered saline (pH 7.4) composed of 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, and 14.7 mM KH_2PO_4 with a flow rate of 0.4 mL min^{-1} at 4 ºC. A calibration curve used to estimate the molecular weight of AxyC was constructed using marker proteins (Gel Filtration Calibration Kit, HMW and LMW; Cytiva).

Optimal pH and temperature of AxyC. The optimal pH of AxyC was determined as follows: a 20 μL reaction mixture containing McIlvaine's buffer[27] at pH 4.0–8.0, 2 mM *p*NP α-D-xylopyranoside, and 30 ng purified AxyC was incubated at 20 ˚C for 10 min. Next, 100 μL of 1 M sodium bicarbonate was added to the mixture to terminate the reaction. The concentration of the released *p*NP was determined by measuring the absorbance at 405 nm.

The optimal temperature of AxyC was determined as follows: a 20 μL reaction mixture containing 50 mM sodium acetate buffer (pH 5.5), 2 mM *p*NP α-D-xylopyranoside, and 15 ng purified AxyC was incubated at 35−60 ˚C for 10 min. Next, 100 μL of 1 M sodium bicarbonate was added to the mixture to terminate the reaction. The concentration of the released *p*NP was determined as described above.

Substrate specificity of AxyC. The substrate specificity of AxyC for *p*NP substrates was examined as follows: a 20 μL reaction mixture containing 2 mM *p*NP substrate (*p*NP α-D-xylopyranoside, *p*NP α-D-glucopyranoside, *p*NP α-D-galactopyranoside, *p*NP α-D-mannopyranoside, *p*NP α-L-arabinopyranoside, *p*NP α-L-arabinofuranoside, *p*NP α-L-fucopyranoside, *p*NP α-L-rhamnopyranoside, *p*NP β-D-xylopyranoside, *p*NP β-D-glucopyranoside, *p*NP β-D-galactopyranoside, *p*NP β-D-mannopyranoside, *p*NP β-L-fucopyranoside, *p*NP β-L-arabinopyranoside, or *p*NP β-D-fucopyranoside), 50 mM sodium acetate buffer (pH 5.5) and 10 ng purified AxyC was incubated at 45 ˚C for 5 min. Next, 100 μL of 1 M sodium bicarbonate was added to the mixture to terminate the reaction. The concentration of the released *p*NP was determined as described above.

The substrate specificity of AxyC for isoprimeverose was determined as follows: a 10 μL reaction mixture containing 5 mM isoprimeverose, 50 mM sodium acetate buffer (pH 5.5), and 200 ng purified AxyC was incubated at 45 ˚C for 10 min. Next, the mixture was incubated at 98 ˚C for 10 min to terminate the reaction. The concentration of released D-glucose was determined using LabAssay Glucose (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

The kinetic parameters of AxyC for *p*NP α-D-xylopyranoside were determined as follows: the 20 μL reaction mixture containing 0.0625−4 mM *p*NP α-D-xylopyranoside, 50 mM sodium acetate buffer (pH 5.5), and 4 ng purified AxyC was incubated at 45 ˚C for 5 min. Next, 100 μL of 1 M sodium bicarbonate was added to the mixture to terminate the reaction. The concentration of the released *p*NP was determined as described above.

Kinetic constants (K_m and k_{cat}) were calculated by nonlinear least-squares data fitting methods using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) as reported previously.[28]

Effects of additives on AxyC activity. The effects of the various additives were examined as follows: a 20 μL of reaction mixture containing additives (4 mM $ZnSO_4$, 4 mM $CuSO_4$, 4 mM $MnCl_2$, 4 mM $FeCl_2$, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 8 mM ethylenediaminetetraacetic acid (EDTA), 10 or

25 % dimethyl sulfoxide (DMSO), 10 or 25 % ethanol, 40 mM D-xylose, 40 mM D-glucose, 40 mM D-galactose, or 40 mM cellobiose), 2 mM *p*NP α-D-xylopyranoside, 50 mM sodium acetate buffer (pH 5.5), and 10 ng purified AxyC was incubated at 45°C for 5 min. Next, 100 μL of 1 M sodium bicarbonate was added to the mixture to terminate the reaction. The concentration of the released *p*NP was determined as described above.

Nucleotide sequence accession number. The cDNA sequence of *axyC* was deposited in DDBJ/EMBL/GenBank under the accession number LC761854.

RESULTS

Identification of novel α-xylosidase in A. oryzae.

Previously, we screened genes that were upregulated in the presence of xyloglucan oligosaccharides or D-xylose in *A. oryzae* and identified β-galactosidase LacA and intracellular α-xylosidase AxyA.[13)14)] Both LacA and AxyA are important for the degradation of xyloglucan oligosaccharides in *A. oryzae*. In addition, two putative genes, AO090005000768 and AO090005000767, were induced in the presence of xyloglucan oligosaccharides or D-xylose (data not shown). These putative genes were located on chromosome 1 and were contiguous within the genome (Fig. 1). To identify the functions of these genes, we amplified their full-length cDNA by PCR, using cDNA synthesized from the total mRNA of *A. oryzae* as a template. The DNA fragment of AO090005000767 was successfully amplified from cDNA using primers 3 and 4 (Fig. 1); however, we could not amplify AO090005000768 from cDNA using primers 1 and 2 (Fig. 1). Next, we tried to amplify DNA fragment from AO090005000768 to AO090005000767 using cDNA of *A. oryzae* as a template and primers 1 and 4. Interestingly, a DNA fragment of approximately 2 kbp was amplified by PCR using primers (primers 1 and 4) and cDNA as a template. The amplified DNA fragment was cloned into the pGAPZ vector as described in Materials and Methods, and the sequence of the DNA fragment was confirmed by sequencing. We found that AO090005000768 and AO090005000767 are connected by splicing. There was an intron in AO090005000768, and the putative stop codon of AO090005000768 predicted in the database did not exist (Fig. 1). Since subsequent analyses revealed that this gene encodes α-xylosidase, we termed this gene as *axyC* (alpha-xylosidase C). The *axyC* gene consists of 2,029 bp and one intron (64 bp), and the length of the open reading frame is 1,965 bp. The deduced protein (AxyC) consisted of 654 amino acid residues.

AxyC does not contain a putative N-terminal signaling peptide for secretion, suggesting that it is an intracellular enzyme. AxyC showed low sequence similarity to *A. oryzae* intracellular α-xylosidase AxyA (identity: 28.8 %) and extracellular α-xylosidase AxyB (identity: 33.4 %), but the putative catalytic nucleophile (Asp382 of AxyC) and acid/base (Asp452 of AxyC) residues were conserved in AxyA, B, and C (Fig. 2). There are no *N*-glycosylation sites in the protein.

Preparation of purified AxyC by heterologous expression in yeast.

Recombinant His_6-tagged AxyC was expressed in *P. pastoris* cells and purified from the cell extract, as described in Materials and Methods. Purified AxyC was analyzed by SDS-PAGE (Fig. 3A). Based on its amino acid sequence, the molecular mass of His_6-tagged AxyC was estimated to be 75.9×10^3 and was approximately consistent with the results of the SDS-PAGE analysis.

In the size exclusion chromatography analysis, the molecular weight of purified AxyC was approximately 288×10^3, indicating that AxyC is a homotetrameric enzyme (Fig. 3B).

Enzymatic characterization and substrate specificity of AxyC.

The substrate specificity of AxyC for various *p*NP substrates was determined using purified recombinant AxyC. The *p*NP substrates used in the present study are described in the Materials and Methods section. Among these, AxyC showed hydrolytic activity toward *p*NP α-D-xylopyranoside, but no detectable activity was observed for the other *p*NP substrates. This result indicated that AxyC is an α-xylosidase. The optimal pH and temperature of AxyC toward *p*NP α-D-xylopyranoside were pH 5.5 and 45 °C, respectively. *A.*

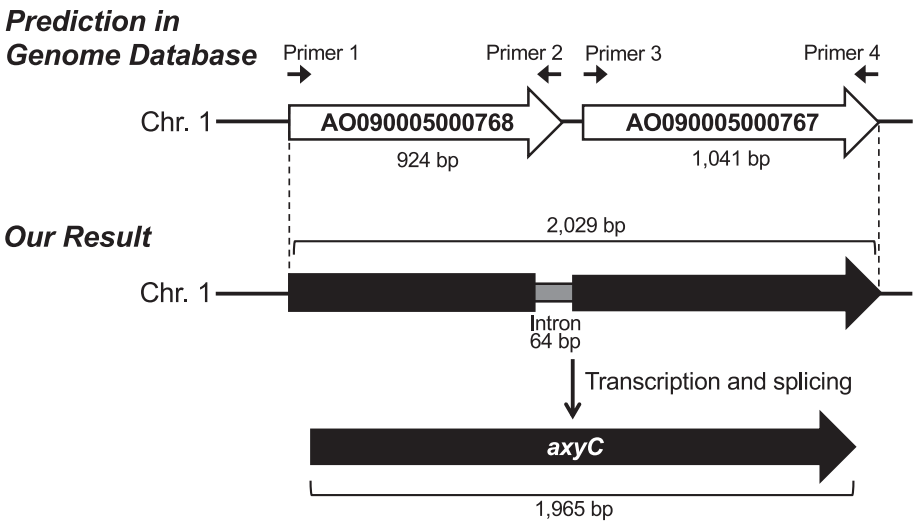

Fig. 1. Gene arrangement of *axyC* in *A. oryzae* genome.

Fig. 2. Sequence alignment of three *A. oryzae* α-xylosidases.

Amino acid sequences of *A. oryzae* AxyA, B, and C were aligned using the Clustal Omega program.[36] Fully conserved, strongly conserved, and weakly conserved amino acid residues are indicated by asterisks, colons, and periods, respectively. N-terminal signal peptide of AxyB for secretion is underlined. Putative catalytic residues are surrounded by black squares.

oryzae AxyA and AxyB showed much higher hydrolytic activity toward isoprimeverose than toward *p*NP α-D-xylopyranoside.[14][15] In contrast, AxyC showed higher hydrolytic activity toward *p*NP α-D-xylopyranoside, but its specific activity toward isoprimeverose was only 1/954 of that toward *p*NP α-D-xylopyranoside (Table 1). The Michaelis constant (K_m) and turnover number (k_{cat}) of AxyC for *p*NP-D-xylopyranoside were 0.67 ± 0.03 mM and 186 ± 2

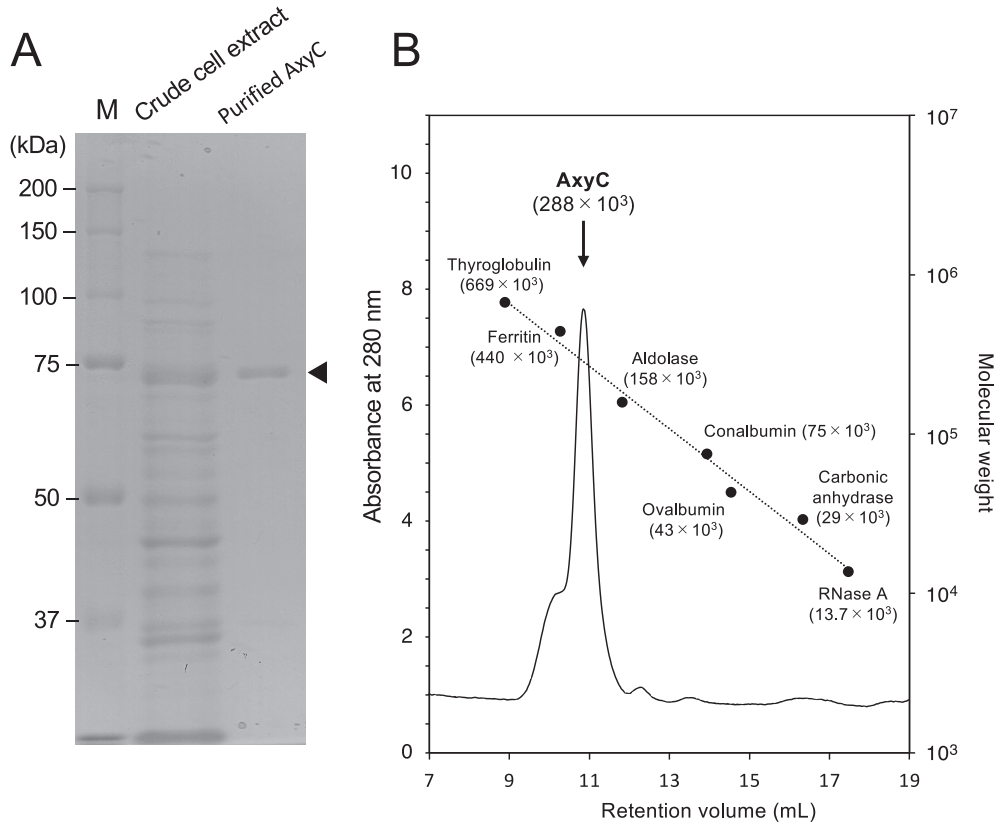

Fig. 3. Purification of heterologously expressed AxyC.

(A) SDS-PAGE analyses of the crude cell extract of *P. pastoris* cells expressing AxyC and purified AxyC. Black triangle represents protein band of AxyC. M: molecular marker. (B) Gel filtration chromatography elution profile of AxyC. Calibration curve used to estimate molecular weight of AxyC is indicated by a dashed line. Dots indicate molecular weight of marker proteins: thyroglobulin (669×10^3), ferritin (440×10^3), aldolase (158×10^3), conalbumin (75×10^3), ovalbumin (43×10^3), carbonic anhydrase (29×10^3), and ribonuclease A (RNase A; 13.7×10^3).

Table 1. Substrate specificity of AxyC.

Substrate	Activity (μmol/min/mg)
*p*NP α-D-xylopyranoside	103 ± 4
Isoprimeverose	0.108 ± 0.008

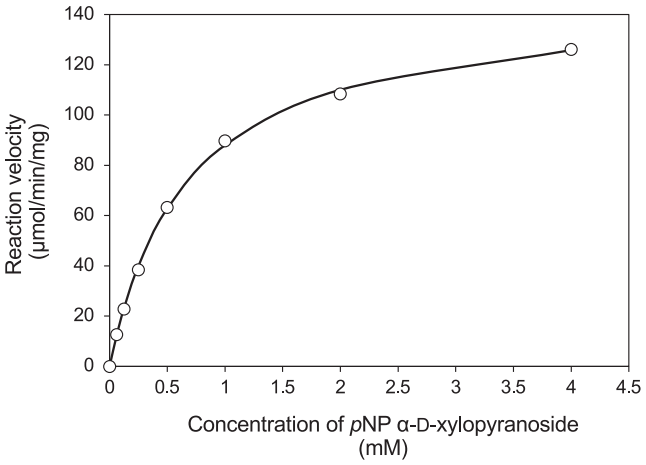

Fig. 4. Kinetic analysis of AxyC.

Plot of substrate concentration versus reaction velocity of AxyC for *p*NP α-D-xylopyranoside is shown.

s^{-1}, respectively (Fig. 4). We also examined whether AxyC releases D-xylose residues from xyloglucan oligosaccharides (XXXG: Glc_4Xyl_3, XLXG/XXLG: $Glc_4Xyl_3Gal_1$, and XLLG: $Glc_4Xyl_3Gal_2$) using thin-layer chromatography; however, the release of D-xylose was not detected (data not shown).

Table 2. Effects of metal ions, organic solvents, and sugars toward AxyC activity.

Additives	Relative activity (%)
No additive	100 ± 7
4 mM $ZnSO_4$	< 5
4 mM $CuSO_4$	< 5
4 mM $MnCl_2$	96.7 ± 7.2
4 mM $FeCl_2$	114 ± 3
4 mM $CaCl_2$	116 ± 10
4 mM $MgCl_2$	99.3 ± 3.0
8 mM EDTA	102 ± 3
8 % DMSO	94.1 ± 0.8
20 % DMSO	73.4 ± 4.0
8 % Ethanol	93.5 ± 4.5
20 % Ethanol	44.9 ± 3.2
40 mM Xylose	84.8 ± 4.5
40 mM Glucose	97.0 ± 2.8
40 mM Galactose	97.2 ± 6.4
40 mM Cellobiose	101 ± 3

Effects of metal ions, organic solvents, and sugars on AxyC activity.

Next, we investigated the effects of metal ions, organic solvents, and sugars on the activity of AxyC (Table 2). The

Table 3. Kinetic parameters of *A. oryzae* α-xylosidases.

Substrate	Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	Reference
*p*NP α-D-xylopyranoside	AxyC	0.67 ± 0.03	186 ± 2	278	This study
	AxyA	1.62 ± 0.24	0.429 ± 0.022	0.265	(14)
	AxyB	–	–	–	–
Isoprimeverose	AxyC	–	–	–	–
	AxyA	0.201 ± 0.051	3.47 ± 0.19	17.3	(14)
	AxyB	5.6 ± 0.4	51.5 ± 1.9	9.20	(15)

–: not determined.

hydrolytic activity of AxyC toward *p*NP α-D-xylopyranoside was almost completely abolished in the presence of zinc and copper ions. Other metal ions and EDTA did not inhibit or activate AxyC, suggesting that metal ions are unnecessary for AxyC. High concentrations of organic solvents (20 % DMSO and ethanol) inhibited AxyC activity. In addition, D-xylose weakly inhibited the hydrolytic activity of AxyC toward *p*NP α-D-xylopyranoside.

DISCUSSION

Recently, the subfamily classification of GH31 using sequence similarity networks and structural analyses was reported.[24] GH31 enzymes are classified into twenty subfamilies (GH31_1 to GH31_20), and α-xylosidases are found in subfamilies 1, 3, 4, and 5. Based on this subfamily classification, the three *A. oryzae* GH31 α-xylosidases, AxyA, AxyB, and AxyC, were classified as subfamilies 3, 5, and 4, respectively. α-Xylosidases derived from eukaryotes (fungi and plants) that have been characterized are subfamilies 1, 3, or 5, and AxyC was the first characterized GH31_4 enzyme in eukaryotes. In archaea and bacteria, *Saccharolobus solfataricus* (SsXylS),[29] *Bacteroides ovatus* (BoYicI_5 and 7),[30][31] *Cellvibrio japonicus* (CjXyl31A),[25] and *Xanthomonas citri* (XylS)[32] produce α-xylosidase belonging to GH31_4, and this subfamily are highly specific toward xyloglucan oligosaccharides.[24] Some bacterial GH31_4 α-xylosidases, such as CjXyl31A and BoYicI_5, have a PA14 domain that facilitates the binding of large xyloglucan oligosaccharides,[25][33] but we did not find the PA14 domain in AxyC.

Both AxyA and AxyC are intracellular α-xylosidases, but there is a clear difference in the substrate specificity. The k_{cat}/K_m value of AxyC for *p*NP α-D-xylopyranoside was approximately 1,000-fold higher than that of AxyA (Table 3). In contrast, the K_m value of AxyA for isoprimeverose is low, suggesting that AxyA is involved in the degradation of isoprimeverose.[14] Because AxyC showed higher hydrolytic activity toward an artificial α-xylopyranosyl substrate (*p*NP α-D-xylopyranoside) but not toward isoprimeverose and xyloglucan oligosaccharides (XXXG, XLXG/XXLG, and XLLG), the candidate substrate(s) of AxyC in nature remain unclear. Previously, we identified a metagenomic α-xylosidase (MeXyl31) from the soil metagenome, which showed higher hydrolytic activity for *p*NP α-D-xylopyranoside, but not for isoprimeverose and xyloglucan oligosaccharides.[34][35] This metagenomic α-xylosidase MeXyl31 also belongs to GH31_4, suggesting that not only isoprimeverose and xyloglucan oligosaccharides used in this study but also other unknown α-xylopyranosyl substrates (oligosaccharide and/or glycoside) could be hydrolyzed by GH31_4 α-xylosidases. Orthologs of AxyC (putative GH31_4 enzymes) are conserved in other *Aspergillus* species, such as *Aspergillus flavus*, *Aspergillus minisclerotigenes*, *Aspergillus nidulans,* and *Aspergillus niger*, and other *A. oryzae* strains (e.g., UniProt ID: I7ZQC4 and A0A1S9DVS8, UniProt: https://www.uniprot.org). Filamentous fungi produce various types of glycosidases to degrade and assimilate polysaccharides and glycosides, but the diversification and functionalization of GH31 α-xylosidases in fungi remain unclear.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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