A novel isoprimeverose-producing enzyme from *Phaeoacremonium minimum* is active with low concentrations of xyloglucan oligosaccharides

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Xyloglucan is one of the major polysaccharides found in the plant cell wall and seeds. Owing to its complex branched structure, several different hydrolases are required to degrade it. Isoprimeverose-producing enzymes (IPase) are unique among the glycoside hydrolase 3 family in that they recognize and release a disaccharide from the nonreducing end of xyloglucan oligosaccharides. Only two IPases have been previously isolated and characterized. A novel IPase from *Phaeoacremonium minimum* (PmIPase) was expressed and characterized. The xylopyranosyl residue at the nonreducing end of xyloglucan oligosaccharides was essential for hydrolytic activity, and PmIPase was unable to hydrolyze cellobiose into D-glucose. PmIPase had a \( K_m \) for xyloglucan oligosaccharide substrate that was much lower than that of the reported IPase isolated from *Aspergillus oryzae*. This indicates that PmIPase was able to produce isoprimeverose efficiently from low concentrations of xyloglucan oligosaccharides. PmIPase also exhibited transglycosylation activity and was able to transfer isoprimeverose units to its substrates.

Xyloglucan plays important roles in plant growth and development and is one of the major polysaccharides found in the plant cell wall and seeds [1]. Xyloglucan is composed of a \( \beta-1,4 \)-linked glucan backbone with glucopyranosyl moieties modified with \( \alpha-1,6 \)-linked xylopyranosyl residues. Some of these xylopyranosyl residues are modified with additional saccharides, such as D-galactose and L-fucose [2]. The structure of xyloglucan can be given as a series of abbreviations, where \( G \) represents an unbranched \( \beta-D \)-glucopyranosyl residue, \( X \) is an \( \alpha-D \)-xylopyranosyl-(1\( \rightarrow \)6)\( \beta-D \)-glucopyranosyl segment, and \( L \) is a \( \beta-D \)-galactopyranosyl-(1\( \rightarrow \)2)\( \alpha-D \)-xylopyranosyl-(1\( \rightarrow \)6)\( \beta-D \)-glucopyranosyl segment (Fig. 1) [3]. Because of its complex branched structure, xyloglucan is degraded by several hydrolases. For example, xyloglucanases are xyloglucan-specific endo-\( \beta \)-glucanases [4–8], and oligoxyloglucan reducing-end-specific celllobiohydrolases release two glucosyl main-chain residues [9]. \( \alpha \)-Xylosidases [10–12], isoprimeverose-producing enzymes (IPase) [13–15], and \( \beta \)-galactosidases [16] are also involved in xyloglucan degradation. Previously, it was reported that some commercial enzymes, such as Driselase (Sigma-Aldrich, St. Louis, MO, USA) [17], and culture supernatant of *Aspergillus oryzae* [18] had isoprimeverose-producing enzymatic activities. Only two IPases have been isolated, one from a bacterium

Abbreviations
CBM, cellulose-binding module; GH, glycoside hydrolase family; G, unbranched \( \beta-D \)-glucopyranosyl residue; IPase, isoprimeverose-producing enzyme; L, \( \beta-D \)-galactopyranosyl-(1\( \rightarrow \)2)\( \alpha-D \)-xylopyranosyl-(1\( \rightarrow \)6)\( \beta-D \)-glucopyranosyl segment; XXXGol, reduced XXXG substrate; X, \( \alpha-D \)-xylopyranosyl-(1\( \rightarrow \)6)\( \beta-D \)-glucopyranosyl segment.
(Oerskvia sp. Y1) [13] and the other from a eukaryote (A. oryzae) [15]. The A. oryzae IPase, called IpeA, releases isoprimeverose (α-D-xylopyranosyl-(1→6)-D-glucopyranose) units from xyloglucan oligosaccharides. The xylopyranose residue at the nonreducing end of xyloglucan oligosaccharide is essential for IpeA activity, and galactosylation of xylopyranose reduces IpeA activity [15]. IPases exhibit both hydrolytic and transglycosylation activities and are able to produce di- and oligosaccharides [13,15]. Based on amino acid sequences, IPases belong to glycoside hydrolase family 3 (GH3; carbohydrate-active enzymes database, http://www.cazy.org/). Most GH3 enzymes recognize and release monosaccharides from the nonreducing end of substrates. However, IPases recognize and release disaccharides. Despite the unique enzymatic properties of IPases, only two such enzymes have been isolated and characterized. The characterization of novel IPases may lead to a better understanding of xyloglucan degradation and GH3 enzymes. In addition, IPases can potentially produce unique oligosaccharides from lignocellulosic biomass. Xyloglucan oligosaccharides have been reported to have biological activities for lipid metabolism, ultraviolet-induced immune suppression, and other processes [19,20]. Production of xyloglucan oligosaccharides, including isoprimeverose, is expected to contribute to the discovery of novel applications of xyloglucan oligosaccharides and effective utilization of lignocellulosic biomass. In this study, we expressed and characterized a novel IPase from Phaeoacremonium minimum (also called Togninia minima). Phaeoacremonium minimum belongs to Sordariomycetes and is frequently isolated from diseased woody plants [21]. Phaeoacremonium minimum isoprimeverose-producing enzyme (PmIPase) hydrolyzes and releases isoprimeverose from the nonreducing end of xyloglucan oligosaccharides. The $K_m$ of PmIPase for a reduced XXXG substrate (XXXGol) was approximately one-thirteenth that of A. oryzae IpeA, and PmIPase was able to produce isoprimeverose at low concentrations of substrate.

Materials and methods

Materials

Xyloglucan oligosaccharides were prepared as described previously [15]. Figure 1 shows the structures of the xyloglucan oligosaccharides used in this study. Tamarind seed xyloglucan was purchased from Megazyme (Wicklow, Ireland), cellobiose from Sigma-Aldrich, and xylobiose from Wako Pure Chemical Industries (Osaka, Japan).

Expression and purification

The gene encoding PmIPase was synthesized by GENE-WIZ (South Plainfield, NJ, USA) with codon optimization for Pichia pastoris, and the synthesized DNA sequence was deposited in DDBJ/EMBL/GenBank under the accession number LC333973. The synthesized PmIPase gene was digested with EcoRI and NotI and ligated to a pGAPZa A vector (Invitrogen, Carlsbad, CA, USA) digested with EcoRI and NotI. pGAPZa A-PmIPase-Myc- and His6-tag vector was treated with AvrII and introduced into P. pastoris strain X-33. Pichia pastoris X-33 cells harboring PmIPase-Myc- and His6-tag were cultured in YPD medium (2% peptone, 1% yeast extract, and 2% d-glucose) containing 50 mM potassium phosphate buffer (pH: 6.0) at 30 °C, 100 r.p.m. for 3 days. After cultivation, cells were removed by centrifugation (5000 g, 15 min) and filtration (0.22 μm). Recombinant PmIPase was purified using a Ni$^{2+}$-affinity column (HisTrap FF; GE Healthcare, Buckinghamshire, UK). The culture supernatant was run through a HisTrap FF column and washed several times with buffer containing 20 mM sodium phosphate (pH: 7.4), 300 mM NaCl, and 10 mM imidazole. The recombinant PmIPase was eluted with buffer containing 20 mM sodium phosphate (pH: 7.4), 300 mM NaCl, and 500 mM imidazole. The purified enzyme was concentrated using a Vivaspin 20–10k cut (GE Healthcare) ultrafiltration system, and the protein concentration was measured by UV (280 nm) absorbance using a NanoDrop spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) and revised using ProtParam (http://web.expasy.org/portparam/). Briefly, the protein concentration measured using the NanoDrop spectrophotometer was divided by the extinction coefficient from ProtParam. Recombinant A. oryzae IpeA was expressed and purified as described previously [15].

Endoglycosidase treatment

Purified recombinant PmIPase was treated with endoglycosidase H (endo H; New England Biolabs, Ipswich, MA, USA) following a slightly modified version of the method described previously [15]. Four micrograms of purified recombinant PmIPase was denatured at 98 °C for 10 min in the presence of 0.5% SDS and 40 mM DTT. Then, 50 mM sodium phosphate buffer (at final concentration, pH: 6.0) and 1000 units of endo H were added to the reaction mixture. The reaction mixture was incubated at 37 °C for 60 min.

Optimal temperature and pH

The optimal pH range of PmIPase was determined as described below. Twenty microliters of reaction mixture containing 0.3 μg of purified recombinant PmIPase, McIlvaine’s buffer [22], and 7.5 mM XXXGol [13,23] was
incubated at 60 °C for 5 min. The resulting reducing sugars were measured using a dinitrosalicylic acid (DNS) reagent method [24]. The optimal temperature of PmlPase activity was determined as described below. Twenty microliters of reaction mixture containing McIlvaine’s buffer (pH: 3.5), 0.3 μg of purified recombinant PmlPase, and 7.5 mM XXXGol was incubated at temperatures between 40 and 65 °C for 5 min. The released sugars were measured using the DNS method described above. The thermostability of PmlPase was determined by incubating the enzyme (0.06 mg/mL) at 40–65 °C in 50 mM sodium acetate buffer (pH: 4.0) for 5 min. After the heat treatment, 12.5 mM XXXGol was added and the reaction mixture was incubated at 60 °C for 5 min. The residual activity of heat-treated PmlPase was measured using the DNS methods, as described above.

Substrate specificity

Aliquots of the reaction mixture (50 μL) containing oligosaccharides (XG, LG, XX, XXX, XXXG, XLLL, XXXGol, cellobiose, and xylobiose), 50 mM sodium acetate buffer (pH: 4.0), and 0.1 μg of purified recombinant PmlPase were incubated at 60 °C for 5 min. To stop the reaction, mixtures were incubated at 98 °C for 10 min. The released isoprimeverose, α-glucose, and α-xylose were measured by HPLC system driven by a pump (PC-2080; JASCO, Tokyo, Japan) and equipped with a refractive index detector (RI-2031; JASCO) using an Aminex HPX-87H 300 × 7.8-mm column (Bio-Rad, Hercules, CA, USA) as described previously [15].

Kinetic analysis of recombinant PmlPase

The kinetic parameters (K_m, k_cat, and k_cat/K_m) of recombinant PmlPase for XXXGol were determined as described below. Twenty microliters of reaction mixture containing 50 mM sodium phosphate buffer (pH: 4.5), purified recombinant enzyme (1 ng of PmlPase or 0.5 ng of IpeA), and XXXGol (0.0125–0.8 mM for PmlPase; 0.0625–4 mM for IpeA) was incubated at 60 °C for 5 min. To stop the reaction, the mixture was incubated at 98 °C for 10 min. The resulting reducing sugars were measured using a bicinchoninate assay [25]. A standard curve was constructed using isoprimeverose. Kinetic constants were calculated using a nonlinear regression of the Michaelis–Menten equation in GRAPHPAD PRISM version 5.0 (GraphPad Software, La Jolla, CA, USA).

Fig. 1. Structures and abbreviations of the xyloglucan oligosaccharides used in this study. (A) XLLG, (B) XXXG, (C) XXX, (D) XX, (E) XG, and (F) LG. Glc, α-glucopyranosyl residue; Xyl, α-xylopyranosyl residue; Gal, α-galactopyranosyl residue. Abbreviations are shown in boldface. In this paper, ‘nonreducing end’ or ‘reducing end’ indicates the nonreducing end or reducing end of the main chain of a xyloglucan oligosaccharide, respectively.
Transglycosylation activity

The transglycosylation activity of PmIPase was examined as described below. Fifty microliters of a reaction mixture containing 8 mM XXXG, 50 mM sodium acetate buffer (pH: 4.5), and 2 μg of purified recombinant PmIPase was incubated at 60 °C for 5 min. To stop the reaction, the mixture was incubated at 98 °C for 10 min. The reaction products were analyzed with an HPLC system driven by a pump (LC-20AD; Shimadzu, Kyoto, Japan) and equipped with a refractive index detector (RID-20A; Shimadzu) using a TSKgel Amide-80 5 μm column (4.6 mm I.D. × 25 cm; Tosoh, Tokyo, Japan), with 60% acetonitrile as the column eluent at a flow rate of 0.8 mL·min⁻¹ at 40 °C.

Mass spectrometry

The mass spectra were acquired using a MALDI-TOF mass spectrometer (Ultraflex TOF/TOF; Bruker Daltonik, Bremen, Germany). Ions were generated using a pulsed 337-nm nitrogen laser and were accelerated to 23.5 kV. All spectra were obtained in the linear mode with a delayed extraction of 60 ns. For sample preparation, 0.5 μL of a matrix solution prepared by dissolving sodium 2,5-dihydroxybenzoate (1 mg·mL⁻¹) and 2,5-dihydroxybenzoic acid (19 mg·mL⁻¹) in 30% ethanol was spotted onto a target plate (MTP 384 target plate ground steel; Bruker Daltonik) and dried. Subsequently, an aliquot (0.5 μL) of the glycan solution was spotted onto the matrix crystal and dried.

Results

Expression and purification of P. minimum IPase

Phaeoacremonium minimum IPase showed homology with A. oryzae IPase IpeA with an identity of 65% [15] and a weak homology with Oerskovia sp. IPase (identity 33%; Fig. 2). Only Oerskovia sp. IPase had a cellulose-binding module family 6 (CBM6), whereas the fungal IPases, PmIPase, and IpeA lack a CBM. Putative catalytic residues, Asp-299 (nucleophile) and Glu-523 (acid/base) residues of PmIPase, were conserved among these IPases.

Phaeoacremonium minimum isoprimeverose-producing enzyme expressed in P. pastoris and purified as described above. The gene encoding PmIPase consisted of 2334 bp and translated to PmIPase consisting of 777 amino acids. Based on an amino acid sequence analysis using SIGNALP 4.1 (www.cbs.dtu.dk/services/SignalP), PmIPase was predicted to have an N-terminal 24-amino-acid signal peptide (Fig. 2). The molecular mass of Myc- and His₆-tagged PmIPase (without the predicted N-terminal signal peptide) was calculated to be 85 kDa. However, SDS/PAGE analyses indicated a molecular mass of approximately 101 kDa for purified recombinant PmIPase (Fig. 3). The protein band of purified PmIPase was moved to approximately 92 kDa by treatment with endo H, indicating that the PmIPase expressed in P. pastoris was N-glycosylated. The optimal pH range and temperature of purified PmIPase toward XXXGol were pH 4.0–4.5 and 60 °C, respectively. Thermostability experiments indicated that the purified recombinant PmIPase was stable at temperatures lower than 60 °C, but was denatured at 65 °C (data not shown).

Substrate specificity of PmIPase

The substrate specificity of purified PmIPase toward various xyloligoclan oligosaccharides, such as XG, XX, and XXXG; cellobiose; and xylobiose, was examined (Table 1). PmIPase showed hydrolytic activity toward XG but not toward cellobiose, indicating that xylose residues at the nonreducing end of oligosaccharides are essential for the hydrolytic activity of PmIPase. PmIPase did not hydrolyze LG, indicating that galactosylation of the xylose residue at the nonreducing end abolishes the PmIPase activity. PmIPase showed almost the same hydrolytic activities toward 4 mM XG and XX. In the case of 2 mM XG and XX, XX was only slightly preferred over XG, but in the case of 8 mM XG and XX, XG was only slightly preferred over XX (Table 1). In a solution containing 4 mM substrate, PmIPase exhibited approximately twofold higher hydrolytic activity toward XG than toward XXXG, and galactosylation of the xylopyranosyl residue at the second glucopyranosyl residue from the nonreducing end did not show any negative effect on PmIPase activity (Table 1).

The hydrolytic activity of PmIPase toward XG increased with increasing substrate concentration (Table 1). By contrast, its hydrolytic activities toward XXXG and XLLG decreased with increasing substrate concentration. For example, the hydrolytic activity of PmIPase toward XXXG at a concentration of 8 mM was less than half of that at a concentration of 2 mM (Table 1).

Kinetics of PmIPase

The kinetic parameters of PmIPase with XXXGol were compared with those of A. oryzae IpeA (Table 2). The kcat of PmIPase was about one-sixth that of A. oryzae IpeA, indicating that PmIPase acts on its oligosaccharide substrates relatively slowly. However, the Km of PmIPase was one-thirteenth that of IpeA, and the kcat/ Km of PmIPase was about 2.3-fold higher than that of...
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\begin{verbatim}
PmIPase -mkgystfasialasvaia-dacV1PvNPATVDDR1ADLLKRM16EDK1AQLQG 59
IpeA  msvgyfktvillglglai-rdekrpYDRPSVPEVEVtD1GLRMK16E53Q5OQG 68
Oerskovia-IPase -----mtttirsrwligivt-lapaAATTQIATGSGATM1P1AEADL 53

PmIPase
ISWINTTGTDFNATG EMNKYRASMFVGVYTNTSISTGNGVIAKLYNHTH1TIGPA 119
IpeA  INWNTTGTTFNLTGEMNKYMGRFVGVYNTSISIADNYKLYNHTH1TIGPA 120
Oerskovia-IPase PFRNPDLPRLEIRKLDLHRDDEESSLLHQPLPVRLGIQWRSTAAAAGHLWTSP 113

PmIPase LVQ5EG1HGFILPANGPFSIPACSWNPELVEKMKGKAIAASLSGVLNM------NEF 172
IpeA  IQTVSLSKGFILGATNENPSIGFACSFNPELIEKMARIGASALSALGVM------HVM 173
Oerskovia-IPase VDG5------VTATATTPFOAVGSLASTTCTGKQVSTVGAGYNGDATPM1WGLN 168

PmIPase AGLQDDGILPLGKRTNEGCRGYICEMAYASVLGQLGN------VAAMRVFAAFT 227
IpeA  CGLQDDGILPLGKRTNEGCRGYICEMAYASVLGQLGN------ISAMRVVEGSP 228
Oerskovia-IPase AGLVNN--DLQDDGILPLGKRTNEGCRGYICEMAYASVLGQLGN------VAAMRVFAAFT 227

PmIPase EGYVNTAPVGHERELLLTILPSKRAIDAGAYTGCAACVYDGAYVAPVDYNLTEL 287
IpeA  EGLNLSPVNGHERELLLTILPSKRAIDAGAYTGCAACVYDGAYVAPVDYNLTEL 288
Oerskovia-IPase ETTLRN---SSSVKVRDEEPDLRRAADDATHIVALVANLINGTIVVAP--SLD 286

PmIPase DSKFQKDFVFLDSAKGDLCNCNFMCFAEDTSEVTSVLPSGNDPAEMGGGSYNYKPTP 347
IpeA  EEMKQKYVWV1SSACADRTAVCFKLVRATISSAVYV1APNADVMEEPSYNYKPTP 347
Oerskovia-IPase SWTDKTELNVESSAPRTNEEQYVAGTDESSLLKAGLDM--MNNDPNQPTIAAVK 345

PmIPase EEMKQKYVWV1SSACADRTAVCFKLVRATISSAVYV1APNADVMEEPSYNYKPTP 347
IpeA  EEMKQKYVWV1SSACADRTAVCFKLVRATISSAVYV1APNADVMEEPSYNYKPTP 347
Oerskovia-IPase SWTDKTELNVESSAPRTNEEQYVAGTDESSLLKAGLDM--MNNDPNQPTIAAVK 345

PmIPase SSVSVAOQCEWSNDSGPEFAIAAEGAG 497
IpeA  ATINAYQAQCWSNDSFGEFAEAAEAKSD 498
Oerskovia-IPase ATVRSITEVRIDPITLDRAAAGAAGAGAAGAAGAAGAAGAAGAAGAAGAAG 523

PmIPase
SAANGKTVGFWSGFDANDQAPNGYVQQLFSVEDRGPDVNLKYSYSGSESEFFPAPSHS 583
IpeA  VAWTVGTNREDEKRLVKONLNAAG 523
Oerskovia-IPase VAWTVGTNREDEKRLVKONLNAAG 523

PmIPase PYYYYVTVSTGNMLASSATADTASEFSGWVLQSTSAVAAAKADAAVAVVVEGTMFINGRE 643
IpeA  VAWTVGTNREDEKRLVKONLNAAG 523
Oerskovia-IPase VAWTVGTNREDEKRLVKONLNAAG 523

PmIPase H1DVA5VZGAPMPVISVDYIIENGTPFPYVFSKGNPVVYPIEWHNTSNAPVQ6FYSEQQ 583
IpeA  HVNVEDLSNLAQPAKLVLILRGNVYVYVWQSLNWIAPVQ6FYSEQ 584
Oerskovia-IPase AVGRNDLPQPKRVELEHAANPRLMLEQDSTOLDQKPVPLWTNTHGQET 783

PmIPase MALSADVFGGDWPSGSDKSVEFSPFDVDGTEFFYLDYNLSAARWPMPGHAYPNTGELVESMNV 643
IpeA  MALSADVFGGDWPSGSDKSVEFSPFDVDGTEFFYLDYNLSAARWPMPGHAYPNTGELVESMNV 643
Oerskovia-IPase HAADVFGGDWPSGSDKSVEFSPFDVDGTEFFYLDYNLSAARWPMPGHAYPNTGELVESMNV 643

PmIPase LNTPLPLLKNCVVTSCSELTSTASSSTLSIVSVYDNYN--NSYEEGTVN 782
IpeA  LNGTHPAWPCNLCSGSESNTLGVAAWDAKLVTNTAEELLVTSDVYDNTKATDA 784
Oerskovia-IPase YGGTTPPALQCVTSCSELTSTASSSTLSIVSVYDNYN--NSYEEGTVN 887

PmIPase KDLVSSPPVNYKCGKAKIPAIAGAAETKDLIJKVAGDLDWJYKYN--VYVPMNGETIF 761
IpeA  VDASEAVSVPPMLKCGKAKIPAIAGAAETKDLIJKVAGDLDWJYKYN--VYVPMNGETIF 761
Oerskovia-IPase HQGHTSRTDAAKLRPDHLGAPYLIPGLVYRLTSLVSAPKROWTRDMKV135VAGVYVGYV 867

PmIPase CASSADLSNTLTV 777
IpeA  CASSADLSNTLTV 777
Oerskovia-IPase CASSADLSNTLTV 777

PmIPase WLAGEDLNQAKRPATFASVSSRAGTGTITLIREGSNPGSGLY1GVATVSTIDAYTAGT 927
IpeA  WLAGEDLNQAKRPATFASVSSRAGTGTITLIREGSNPGSGLY1GVATVSTIDAYTAGT 927
Oerskovia-IPase WLAGEDLNQAKRPATFASVSSRAGTGTITLIREGSNPGSGLY1GVATVSTIDAYTAGT 927

PmIPase VTSVAHAAHODYYVLKGMTRFRMAO 1018

\end{verbatim}
IpeA. These results indicate that PmIPase had a much higher affinity and catalytic efficiency toward XXXG than A. oryzae IpeA. In this analysis, the specific activity of PmIPase toward 0.2 mM XXXGol substrate was 62.0 ± 2.6 µmol-min⁻¹-mg⁻¹. This is discussed in greater detail below.

Transglycosylation activity of PmIPase

The transglycosylation activity of PmIPase was also examined. PmIPase was incubated in a high concentration (8 mM) of XXXG for 5 min. PmIPase produced not only isoprimeverose, which is a hydrolysis product, but also XXXXG and XXXXXXG (Fig. 4B,C). Transglycosylation of PmIPase was also observed when PmIPase was incubated with XX (data not shown).

Discussion

Isoprimeverose-producing enzymes release isoprimeverose from xyloglucan oligosaccharides and are key enzymes in the production of various oligosaccharides via hydrolysis and transglycosylation. As described above, PmIPase exhibited a high transglycosylation activity in high concentrations of substrate. The observed decrease in hydrolytic activity with
increasing concentrations of XXXG and XLLG may partially result from transglycosylation. The substrate specificity of PmIPase resembled that of \textit{A. oryzae} IpeA. For example, both enzymes recognized the isoprimeverose unit of xyloglucan oligosaccharides at the nonreducing end, with the xylopyranosyl side chain being essential for their hydrolytic activities. Galectosylation of the xylopyranosyl side chain on the glucopyranosyl residue at the nonreducing end of xyloglucan oligosaccharide abolished all hydrolytic activity. Some differences in substrate specificity were evident between PmIPase and \textit{A. oryzae} IpeA. \textit{Aspergillus oryzae} IpeA preferred xyloglucan oligosaccharides containing four glucosyl main-chain residues, such as XXXG, over substrates containing two glucosyl main-chain residues, such as XG and XX. By contrast, PmIPase preferred substrates containing two glucosyl main-chain residues at high substrate concentrations (4–8 mM). As described above, the specific activity of PmIPase toward 0.2 mM XXXGol substrate (0.2 mM is approximately fourfold higher than the $K_m$ of PmIPase) was almost the same as that toward 2 mM XXXGol. In addition, the specific activities toward 4 mM XG or XX were higher than those toward 2 mM XG or XX, respectively. These results suggest that the $K_m$ values for XG and XX were much higher than that for
XXXGol and that PmIPase preferred xyloglucan oligosaccharides containing four glucosyl main-chain residues at low substrate concentrations. The hydrolytic activity of _A. oryzae_ IpeA was inhibited by galactosylation of the second xylopyranosyl residue from the nonreducing end, whereas PmIPase was unaffected. These results suggest differences in the substrate recognition abilities of positive subsites (+1, +1’, +2, etc.) in PmIPase and _A. oryzae_ IpeA. Future studies will focus on crystal structure analyses of IPases to elucidate the mechanisms that allow IPases to recognize xyloglucan oligosaccharide substrates at negative and positive subsites.

Based on a protein BLAST search (https://blast.ncbi.nlm.nih.gov/ Blast.cgi), _P. minimum_ hosts other putative enzymes related to xyloglucan degradation, including GH74 xyloglucanase (NCBI Reference Sequence: XP_007915470.1) and _α_-xylosidase (NCBI Reference Sequence: XP_007915392.1). It is hypothesized that these enzymes act together with PmIPase to hydrolyze and assimilate xyloglucan into _P. minimum_.

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**Author contributions**

TM and KY conceived and designed the experiments. AK analyzed the oligosaccharides. TM performed and analyzed all other experiments. TM, AK, and KY wrote the paper.

**Conflict of interest**

The authors declare no conflict of interest.

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