Identification of the Gene Encoding Isoprimeverose-producing Oligoxyloglucan Hydrolase in Aspergillus oryzae*

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Aspergillus oryzae produces a unique β-glucosidase, isoprimeverose-producing oligoxyloglucan hydrolase (IPase), that recognizes and releases isoprimeverose (α-D-xylopyranose-1→6-D-glucopyranose) units from the non-reducing ends of oligoxyloglucans. A gene encoding A. oryzae IPase, termed ipeA, was identified and expressed in Pichia pastoris. With the exception of cellobiose, IpeA hydrolyzes a variety of oligoxyloglucans and is a member of the glycoside hydrolase family 3. Xylopyranosyl branching at the non-reducing ends was vital for IPase activity, and galactosylation at a α-1,6-linked xylopyranosyl side chain completely abolished IpeA activity. Hepta-oligoxylglucan saccharide (Xyl6Glc3) substrate was preferred over tri- (XylGlc) and tetra- (Xyl2Glc) oligoxyloglucan saccharides substrates. IpeA transferred isoprimeverose units to other saccharides, indicating transglycosylation activity. The ipeA gene was expressed in xylose and xyloglucan media and was strongly induced in the presence of xyloglucan endo-xylloglucan-hydrolized products. This is the first study to report the identification of a gene encoding IPase in eukaryotes.

Plants are composed of various polysaccharides, including cellulose, xylan, and xyloglucan. Xyloglucan is a polysaccharide found in the primary cell wall and seed and has important roles in plant development and growth (1). Xyloglucan has a β-1,4-glucan backbone and α-linked xylopyranosyl residues attached to the C6 position of glucopyranosyl residues in the β-1,4-glucan. In addition, xyloglucan is modified with other sugars, such as galactose, arabinose, and fucose (2). Structures of xyloglucan are designated as follows: X, α-1,6-xylopyranosyl-β-1,4-glucopyranose; L, β-1,2-galactopyranosyl-α-1,6-xylopyranosyl-β-1,4-glucopyranose; and G, unbranched glucopyranosyl residue (3). Xyloglucan polysaccharides are divided into two types, XXXG, XLXG, XXLG, and XLLG (3, 6, 9, 13–17). In addition to endo-xylloglucanases, exo-type enzymes, such as oligoxyloglucan-specific celllobiohydrolases (EC 3.2.1.150) (18, 19) and isoprimeverose-producing oligoxyloglucan hydrolases (IPase) (3.2.1.120) (20–22), are involved in xyloglucan degradation. Oligoxyloglucan-specific celllobiohydrolase release cellobiose units from the reducing ends of oligoxyloglucans. IPases are unique β-glucosidases that hydrolyze the β-1,4-glucan backbone of oligoxyloglucans and release isoprimeverose (α-D-xylopyranose-1→6-D-glucopyranose) from the non-reducing ends of oligoxyloglucans.

Previously, only two IPases had been isolated and characterized as follows: one from the bacterium Oerskovia sp. Y1 (21, 22), and the other from the fungus Aspergillus oryzae (20). Oerskovia IPase hydrolyzes XXXG to isoprimeverose and XXG and produces isoprimeverose and glucose as final products (21, 22). Oerskovia IPase does not hydrolyze cellobiose, and the branching of xylpyranosyl residues at the non-reducing ends is essential for its activity. In these earlier studies, the gene encoding the Oerskovia IPase was identified and expressed in Escherichia coli (22). Oerskovia IPase consists of 1018 amino acids and has a molecular mass of 105 kDa. Oerskovia IPase belongs to the glycoside hydrolase family 3 (GH3) family and has a carbohydrate-binding module (CBM) family 6 at its C terminus (22). Oerskovia IPase binds to tamarind seed xyloglucan but not to crystalline cellulose, indicating that the CBM6 of Oerskovia IPase can bind to xyloglucan (an Oerskovia IPase substrate). The first reported IPase was purified from an A. oryzae commercial enzyme preparation, and it was found that this IPase was able to release isoprimeverose from XXXG (20). The A. oryzae IPase was not enhanced by addition of cations (such as Ca2+ and Mn2+) and was significantly inactivated in the presence of Cu2+ and Hg2+ (20). However, the gene encoding this A. oryzae IPase was not identified.

In this study, we identified the A. oryzae IPase gene, termed ipeA, and expressed it in Pichia pastoris. Although we classified IpeA as a GH3 family member, we found that it had only low similarity to Oerskovia IPase and other characterized GH3 family enzymes. IpeA characteristics, including substrate specificity, kinetic constants for various xyloglucan oligosaccharides, and transglycosylation activity, were determined in our study.

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2 The abbreviations used are: TXG, tamarind seed xyloglucan; IPase, isoprimeverose-producing oligoxyloglucan hydrolase; CBM, carbohydrate-binding module; GH3, glycoside hydrolase family 3; pNP, p-nitrophenol; endo H, endoglycosidase H; OREX, oligoxyloglucan-reducing end-specific xyloglucanohydrolase.

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The results from this study illuminate previously uncharacterized eukaryotic GH3 family enzymes.

**Experimental Procedures**

**Materials**—TXG was purchased from Megazyme (Wicklow, Ireland), and oligo-xyloglucan saccharides were prepared as described previously (18, 21, 23). Briefly, XXXG, XXLG, XLLG, and XLXG were obtained from hydrolysis of TXG by *Streptomyces* endo-glucanase (23). XG, XX, and LG were obtained from hydrolysis of XXXG and XXLG by *Geotrichum* oligoxylo-glan-specific cellobiohydrolase (18). XXXG was obtained from partial hydrolysis of XXXG by *Oerskovia* IPase (21). Oligoxylo-glan-saccharides were fractionated on a Bio-Gel P-4 column (3 × 120 cm) (Bio-Rad) (23). Cellobiose, *p*-nitropheryl (*p*NP)-β-d-cellobioside, *p*NP-β-d-glucopyranoside, and *p*NP-α-d-xylopyranoside were purchased from Sigma. *p*NP-α-d-glucopyrano-side and *p*NP-β-d-xylopyranoside were purchased from Nacalai Tesque (Kyoto, Japan). Xylobiose was purchased from Wako Pure Chemical Industries (Osaka, Japan). Chromogenic substrates were dissolved in water at a final concentration of 20 mM. XXXG substrate (XXXGol), which is reduced by borohy-dride reduction of the reducing ends of XXXG, was prepared as described previously (21, 24).

**Expression and Purification of Recombinant IpeA**—The *ipeA* gene was amplified using primers (5′-AGAGAGGCCGCTAGACCTCGATGGAAGGACTGCTAAG-3′ and 5′-ATGAGGATGGTCGACCTGCACATAGAAAGTAGCATTGCC-3′) and a cDNA template. cDNA was synthesized from *A. oryzae* total RNA. After amplification, the PCR fragment was integrated into a linearized pGAPZ-α-A vector using In-Fusion PCR (Takara Bio, Siga, Japan). The linearized pGAPZ-α-A was amplified using primers (5′-AGCTTCAGGCTCTTTTTCGAGAG-3′ and 5′-GTCGACATTAGATGAAAGTACGCATGCTTGGCC-3′) and a cDNA template. CDNA was synthesized from *A. oryzae* total RNA. After amplification, the PCR fragment was integrated into a linearized pGAPZ-α-A vector using In-Fusion PCR (Takara Bio, Siga, Japan). The linearized pGAPZ-α-A was amplified using primers (5′-AGCTTCAGGCTCTTTTTCGAGAG-3′ and 5′-GTCGACATTAGATGAAAGTACGCATGCTTGGCC-3′) and a cDNA template. The resulting pGAPZ-α-A-*ipeA*-His6 vector was transformed into *P. pastoris* strain X-33. X-33 harboring pGAPZ-α-A-*ipeA*-His6 was grown in YPD medium (1% peptone, 0.5% yeast extract, 2% glucose) containing 100 mM potassium phosphate (pH 6.0) and cultured at 30 °C, 100 rpm for 3 days. After cultivation, cells were harvested, and recombinant His-tagged IpeA was purified from culture supernatant using HisTrap FF crude (GE Healthcare, Buckinghamshire, UK). The protein concentration of purified enzyme was measured by UV (280 nm) absorbance using a NanoDrop spectrophotometer (NanoDrop Technologies, Rockland, DE) and revised using ProtParam. The XXXG hydrolysis activity of each fraction was assayed by measuring the production of reducing sugars from XXXGol (21). A fraction of purified IpeA was treated with endo-glucosidase H (endo H) (New England Biolabs). Briefly, 18 μg of purified IpeA was denatured at 99 °C for 10 min with 0.5% SDS and 40 mM dithiothreitol (DTT). Then 2000 units of endo H and endo H buffer were added, and the reaction mixture was incubated at 37 °C for 60 min.

**Kinetic Analysis of Recombinant IpeA**—The kinetic parameters (*k*ₐₚ and *k*ₐ_,*v*_max) of recombinant IpeA for XG, XX, XXXG, and XLLG were determined at concentrations of 1.25 to 20 mM (XG and XX), 0.5 to 4 mM (XXXG), or 0.5 to 6 mM (XLLG) using 0.05 μg of recombinant IpeA. A 50-μl reaction mixture containing 50 mM sodium acetate buffer (pH 4.5) was incubated at 60 °C for 5 min and then at 98 °C for 10 min to stop the reaction. The concentration of released isoprimeverose, glucose, or xylose was determined using an Aminex HPX-87H 300 × 7.8-mm column and an HPLC system driven by a pump (PC-2080) (Jasco, Tokyo, Japan) and equipped with a refractive index detector (RI-2031) (Jasco); 5 mM sulfuric acid was used as the column eluent at a flow rate of 0.6 ml/min at 65 °C. Substrate specificity for chromogenic substrates was determined in 20 μl of reaction mixture containing 10 μM *p*NP substrates (*p*NP-β-d-glucopyranoside, *p*NP-α-d-glucopyranoside, *p*NP-β-d-cellobioside, *p*NP-α-d-xylopyranoside, and *p*NP-α-d-xylopyranoside), 50 mM sodium acetate buffer (pH 4.5), and 0.1 μg of purified recombinant IpeA and incubated at 60 °C for 5 min. The reaction was stopped by the addition of 50 μl of 1 M NaHCO₃, and the concentration of released *p*NP was determined by absorbance measurement at 405 nm using an Infinite M200 PRO microplate reader (Tecan, Zurich, Switzerland).

**Results**

**Effects of pH and Temperature on IpeA Activity**—The optimal pH for recombinant IpeA activity toward XXXGol was evaluated in McIlvaine’s buffer (pH 3.0–8.0) (25). The total reaction volume was 50 μl. The reaction mixture, containing 2 mM XXXGol, 0.05 μg of IpeA and McIlvaine’s buffer, was incubated at 40 °C for 10 min. The resulting reducing sugars were measured using the 3,5-dinitrosalicylic acid reagent method (26). The optimal temperature for recombinant IpeA toward XXXGol was evaluated in 50-μl reaction mixture containing 2 mM XXXGol, 0.05 μg of IpeA, and 100 mM sodium acetate buffer (pH 4.5). The reaction mixture was incubated at 40–65 °C for 10 min, and the resulting reducing sugars were measured using the 3,5-dinitrosalicylic acid reagent method.

**Transglycosylation Activity**—Recombinant IpeA transglyco-sylation products were analyzed using an HPLC system (pump PC-2080 and refractive index detector RI-2031) (Jasco). A 50-μl
reaction mixture containing 80 mM XX, 50 mM sodium acetate buffer (pH 4.5), and 0.2 µg of recombinant IpeA was incubated at 60 °C for 0, 5, 10, 20, 30, and 60 min. Recombinant IpeA was inactivated by incubation at 98 °C for 10 min. The oligoxyloglucan products of transglycosylation were analyzed using a Superdex Peptide 10/300 GL gel filtration column (GE Healthcare) with a 12.5 mM sodium acetate buffer (pH 4.5) as the column eluent (flow rate of 0.6 ml/min).

Mass Spectrometry—MS spectra were acquired using a MALDI-TOF mass spectrometer (Reflex IV; Bruker Daltonik, Bremen, Germany). Ions were generated by a pulsed 337-nm nitrogen laser and were accelerated to 20 kV. All spectra were obtained in the reflectron mode with a delayed extraction of 200 ns. For sample preparation, 0.5 µl of a matrix solution prepared by dissolving sodium 2,5-dihydroxybenzoate (0.5 mg/ml) and 2,5-dihydroxybenzoic acid (9.5 mg/ml) 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.

Expression Analysis—A. oryzae KBN616 (an industrial shoyu koji mold) and ΔxlnR strains were used for expression analysis (27, 28). TXG was dissolved in 20 mM sodium phosphate buffer (pH 6.0) (at a final concentration of 0.4% (w/v)) and placed on a Thermomixer (Eppendorf, Hamburg, Germany) at 50 °C, 750 rpm, for ~5 h. The catalytic domain (1.2 mg) of endo-xyloglucanase XEG74 (XEG74CD) (13) was added to 50 ml of 0.4% TXG endo-xyloglucanase-hydrolyzed products. After TXG hydrolysis, XEG74CD was inactivated by incubation at 98 °C for 20 min. XEG74CD was expressed in E. coli as described previously (14) and purified using a HiTrap HP column (GE Healthcare). Carbon sources (6% glucose, 0.4% cellobiose, 0.4% xylose, 0.4% TXG, and 0.4% TXG endo-xyloglucanase-hydrolyzed products) were sterilized by filtration (0.45 µm) and mixed with the same volume of 2X Czapek-Dox medium (0.6% NaNO3, 0.4% KCl, 0.2% KH2PO4, 0.1% MgSO4·7H2O, 0.002% FeSO4·7H2O, pH 6.0).

A. oryzae RIB40 strain was pre-cultured in Czapek-Dox medium containing 3% glucose and 1% polypeptide at 30 °C, 140 rpm, for 24 h. Cells were harvested and grown in 20 ml of Czapek-Dox medium containing 3% glucose, 0.2% cellobiose, 0.2% xylose, 0.2% TXG, and 0.2% TXG endo-xyloglucanase-hydrolyzed products as the carbon source. The culture was incubated at 30 °C, 140 rpm, for 5 h. After incubation, cells were harvested, and total RNA was extracted using a RNA Pro Red kit (MP Biomedicals). cDNA was synthesized using 1.2 µg of total RNA and PrimeScript II first strand cDNA synthesis kit (Takara Bio). Reverse transcriptase PCR was used to analyze expression of ipeA, xynF1 (endo-xylanase F1, AO0090103000423, Gene ID, 5999107) (27), xynG2 (endo-xylanase G2, AO0090120000026, Gene ID, 5996057) (27), xylA (β-xylosidase A, AO0090050000986, Gene ID, 5990515) (27, 29), and actA (internal control, AO0090701000065, Gene ID, 5995014). The following primers were used: 5’-ATGGTTTCCGGTGTCTTCTAAGG-3’ and 5’-CTGACATAGAAAGTAGCATTGGC-3’ for ipeA; 5’-ATGGTACACCTTAAGGACATTCGTTGC-3’ and 5’-TTCAGAGCGCATCG-ATGATAGCATTACGC-3’ for xynF1; 5’-ATGGTGTCC-TTCTCCTCCTGCTTCTGG-3’ and 5’-TCAATAACAGTGATACAGAAGACCCAC-3’ for xynG2; 5’-ATGCTCGTG-TGCAGGTCCTACCTGTC-3’ and 5’-CTATTGGCGCGCA-ATCAACTGCTCCTCC-3’ for xylA; and 5’-GTTGTCTGCT-TCGTCACTGACACATTG-3’ and 5’-GAAGCATTGGCGTG- AAAAGATCG-3’ for actA.

Results

Identification, Cloning, and Heterologous Expression of the A. oryzae IPase Gene—The amino acid sequence of purified A. oryzae IPase was determined. Purified IPase was digested with lysyl endopeptidase (Wako Pure Chemical Industries) to remove possible N-terminal modifications. N-terminal amino acid sequences of three peptides (peptides 1–3) were determined. The sequences of peptides 1–3 were LIHTQEAVD-LARELD, FEMGLFENPYNAAPA, and AIIDTGVPTVVVLSS, respectively. Amino acid sequences were analyzed using a BLAST search of the Aspergillus genome (AspGD), and a candidate gene encoding A. oryzae IPase was identified. The candidate gene was AO0090701000274 (AspGD ID, and ASPL0000298768, Gene ID, 5995196), and it was termed ipeA (isoprimeverose-producing enzyme) in this study. The ipeA gene was located on chromosome 5 and consisted of 2959 bp, including 10 introns. The length of the open reading frame was 2337 bp. Translated IPase consisted of 779 amino acids and was predicted to have an N-terminal 22-amino acid signal peptide based on amino acid sequence analysis using SignalP 4.1.

Based on the amino acid sequence, IPase was predicted to belong to the GH3 family. GH3 family members include β-glucosidases (EC 3.2.1.21), β-xylosidases (EC 3.2.1.37), β-glucosylceramidase (EC 3.2.1.45), Oerskovia IPase (EC 3.2.1.120), etc. (carbohydrate-active enzymes (CAZy)) (30). The phylogenetic tree of IPase and characterized GH3 enzymes sharing similarity with IPase (NCBI BLAST, blast.ncbi.nlm.nih.gov) are shown in Fig. 1. IPase showed low similarity to Salmonella enterica β-glucosidase BglX (identity, 34%; similarity, 51%), E. coli β-glucosidase BglX (identity, 33%; similarity, 51%), and Ruminoclostridium thermocellum β-glucosidase BglB (identity, 34%; similarity, 51%). Comparison of amino acid sequences between IPase and Oerskovia IPase revealed 35% identity and 52% similarity, indicating the amino acid sequence of IPase is novel. Although Oerskovia IPase has a CBM6 at its C terminus, IPase did not have a CBM. In the phylogenetic tree, IPase showed little similarity to not only β-glucosidases (EC 3.2.1.21) (such as E. coli BglX, S. enterica BglX, R. thermocellum BglB, and A. oryzae BglC) but also to β-glucosidases (EC 3.2.1.37) (e.g. Aspergillus niger XynD, Aspergillus fumigatus XynD, A. oryzae BxlB, and Arabidopsis thaliana Bxl3).

The ipeA gene encoding processed IPase (amino acids 23–779) was cloned into a pGAPZ-α vector and expressed in P. pastoris. His-tagged IPase was purified from a culture supernatant of P. pastoris harboring the IPase expression vector using a nickel-affinity column (Fig. 2). The yield of the purified recombinant IPase was ~7 mg liter−1 of cell culture. Although the molecular mass of His-tagged IPase was estimated to be 84 kDa, the apparent molecular mass of purified His-tagged IPase was ~100 kDa according to SDS-PAGE analysis (Fig. 2, lanes 1 and

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When IpeA was treated with endo H, the protein band was 85 kDa (Fig. 2, lane 4), indicating that purified IpeA was N-glycosylated. The optimal temperature and pH of recombinant IpeA with XXXGol were 60 °C and pH 4.5, respectively.

**Substrate Specificity of Recombinant IpeA**—The specific activities of recombinant IpeA toward various oligopyloglucans and chromogenic substrates were examined (Table 1). IpeA exhibited hydrolytic activity toward XG and produced isopri-
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![Image](FIGURE 2. SDS-PAGE analysis of purified IpeA. Lane M, molecular markers; lane 1, crude enzyme (culture supernatant); lane 2, purified recombinant IpeA. Purified recombinant IpeA was denatured and incubated without or with endo H (lanes 3 and 4, respectively) at 37 °C for 1 h.)

![Image](FIGURE 3. Subsite image of A. oryzae IPase with Xyl3Glc4. G, glucopyranosyl residue; X, xylopyranosyl residue. The arrow indicates the site of cleavage.)

TABLE 1
Substrate specificity of IpeA

| Substrates       | Activity μmol/min/mg | Relative activity % |
|------------------|----------------------|---------------------|
| Oligoxyloglucan substrate (5 mM) |                      |                     |
| XG               | 226 ± 20              | 100 ± 9              |
| LG               | ND                   | ND                   |
| XX               | 307 ± 21              | 136 ± 9              |
| XGX              | 422 ± 19              | 187 ± 8              |
| XXXG             | 489 ± 21              | 217 ± 9              |
| XLXG             | 296 ± 27              | 131 ± 12             |
| XLLG             | 297 ± 18              | 132 ± 8              |
| XXXGol           | 427 ± 30              | 189 ± 13             |
| Cellbiose        | ND                   | ND                   |
| Xylobiose        | ND                   | ND                   |
| TXG (0.4%)       | ND                   | ND                   |
| Chromogenic substrates (10 mM) |                      |                     |
| pNP-β-0-glucopyranoside | 0.23 ± 0.05 | 0.10 ± 0.02 |
| pNP-α-0-glucopyranoside | ND            | ND                   |
| pNP-β-0-xylopyranoside | 1.32 ± 0.06 | 0.59 ± 0.03 |
| pNP-α-0-xylopyranoside | ND            | ND                   |
| pNP-β-0-celllobioside | ND            | ND                   |

of the second glucopyranosyl residue from the non-reducing end (+1 subsite) was important for turnover. The $K_m$ value of IpeA for XXXG was ~2-fold lower than that for XX. The catalytic efficiency constant ($k_{cat}/K_m$) for XXXG was much higher than those for XG, XX, and XLLG. These results indicate that, among these substrates, XXXG was the most preferred IpeA substrate.

Transglycosylation Activity—A. oryzae IpeA released isoprimeverose units from oligoxyloglucans at the non-reducing ends. Next, we examined whether IpeA was able to transfer isoprimeverose units to other oligosaccharides (Fig. 4A). IpeA was incubated with a high concentration (80 mM) of XX. Five to 30 min into the reaction, isoprimeverose, XX, XXX, and XXXX were detected. In addition, a small amount of XXXXX was detected midway through the reaction (20 min). Production of XXX, XXXX, and XXXXX was confirmed by mass spectrometry analysis (Fig. 4B). In XX, a small contaminant of a tetrasaccharide consisting of three hexoses and one pentose ($m/z$ 659, $[H3P1 + Na]^+$), which was not hydrolyzed by IpeA, was detected. These results indicate that IpeA can transfer isoprimeverose to other oligosaccharides. Toward the end of the reaction (60 min), XXX, XXXX, and XXXXX products were hydrolyzed to isoprimeverose.

Expression Pattern of ipa—Next, the expression pattern of the ipa gene was examined (Fig. 5). Previously, ipa gene expression was reported to be controlled by the transcriptional activator XlnR (in that paper, ipa was referred to as AO090701000274) (28). XlnR mediates the expression of many genes, including genes encoding endo-xylanases (XynF1 and XynG2) and β-xylanosidase (XylA), in response to xylan and xylose (27, 28). However, in both A. oryzae wild type (KBN616) and ΔxlnR strains, no detectable expression of ipa was
observed in the glucose or cellobiose medium. The ipeA gene was expressed in the xylose or TXG medium and strongly induced in the presence of TXG endo-xyloglucanase-hydrolyzed products in the wild type strain (Fig. 5). These results indicate that A. oryzae produces IpeA to hydrolyze and utilize oligoxyloglucans as carbon sources. In addition to ipeA, expression of xynF1, xynG2, and xylA was induced not only in the xylose medium but also in the medium of the TXG endo-xyloglucanase-hydrolyzed products (Fig. 5). The induced expression of ipeA, xynF1, xynG2, and xylA in xylose medium was almost completely abolished by deletion of xlnR. However, even in the absence of xlnR, ipeA expression was induced by medium containing TXG or TXG endo-xyloglucanase-hydrolyzed products (Fig. 5).

**Discussion**

Because the characterized GH3 glycoside hydrolases recognize and release individual glycosyl residues from the non-reducing ends of substrates, chromogenic substrates, such as pNP-monosaccharides, and disaccharides have been widely used for screening and functional analyses of GH3 glycoside hydrolases. However, we previously identified and characterized a unique GH3 glycoside hydrolase (Oerskovia IPase) that can release a disaccharide (isoprimeverose) from the non-reducing ends of oligoxyloglucans (21, 22). In A. oryzae, although there are more than 20 genes encoding GH3 family enzymes, including four β-glucosidases (EC 3.2.1.21: BglA, BglF, BglH, and BglJ) (31, 32) and one β-xylosidase (EC 3.2.1.37: XylA) (29).
enzymes involved in lignocellulosic biomass degradation will be of utmost importance.

IpeA recognizes isoprimeverose units at the non-reducing ends of oligoxyloglucans and releases isoprimeverose residues. For example, IpeA hydrolyzes XXXG to X (isoprimeverose) and XXG. The specific activity of IpeA toward XXXGol was much higher than that previously reported for Oerskovia IPase (85 µmol/min/mg) (Table 1) (21). Xylose branching at subsite +1 (second glucopyranosyl residue from the non-reducing end) is not essential for IpeA β-glucosidase activity but contributes to the \( V_{\text{max}} \) value and turnover number. Hydrolyase activity was completely abolished by galactosylation at branched xylpyranosyl residues at the non-reducing ends (subsite −1) and was partially blocked by galactosylation at subite +1. In addition, the \( K_m \) value for XXXG was much lower than that for XX, indicating IpeA has more than three subites (−1, +1, and +2) (Fig. 3). These findings suggest that IpeA has a very unique subsite structure that differs from that of other GH3 family β-glucosidases and β-xyloligosidases. Because cellobiose-utilizing unit-containing oligoxyloglucan substrates, such as XXXG, are products of xylol glucan degradation by endo-xylolucanases, we believe that IpeA and endo-xylolucanases act in coordination to hydrolyze xylol glucan. Previously, an oligoxyloglucan-reducing end-specific xylol glucanobihydrolase (OREX), which belongs to the glycoside hydrolase family 74 (GH74) and releases two glycosyl residue segments (such as XG and LG) from the reducing ends of oligoxyloglucans, was isolated from \( A. \) nidulans (19). Enzymes corresponding to \( A. \) nidulans OREX have not been identified from \( A. \) oryzae. However, synergy among endo-xylolucanase, OREX, IPase, and other enzymes, such as β-galactosidase, might be important for xylol glucan degradation.

IpeA was able to transfer isoprimeverose units to other saccharides, indicating high transglycosylation activity. In this study, we were able to produce unnatural oligosaccharides, XXX, XXXX, and XXXXX, indicating that IpeA has the potential to produce new oligosaccharides by transglycosylation of isoprimeverose units.

Ongoing experiments currently focus on determining the IpeA structure by x-ray crystallography. This analysis will help clarify the mechanism by which IpeA recognizes oligoxyloglu-
can substrates and transfers isoprimeverose units to other saccharides.

**Author Contributions**—T. M., Y. M., and K. Y. conceived and designed the experiments. Y. M. did the purification of IPase. A. K. analyzed the oligosaccharides. T. M. performed and analyzed all other experiments. T. M., A. K., and K. Y. wrote the paper.

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