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Note

Production of Isoprimeverose from Xyloglucan Using *Aspergillus oryzae*

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Abstract: Isoprimeverose [α-D-xylopyranosyl-(1→6)-D-glucose] is produced from xyloglucan using the cooperative action of glycoside hydrolases including isoprimeverose-producing oligoxyloglucan hydrolase and β-galactosidase in *Aspergillus oryzae*. This study investigated *A. oryzae* strains and culture conditions suitable for isoprimeverose production from xyloglucan. Each strain of *A. oryzae* had a different ability to degrade xyloglucans. When an *A. oryzae* strain with high xyloglucan-degradation activity was cultured in a medium containing partially degraded xyloglucan as the carbon source, the production of glycoside hydrolases that degrade xyloglucan into isoprimeverose was highly induced. Our procedure efficiently produced isoprimeverose from xyloglucan without any genetically modified microorganisms or purification of enzymes.

Key words: isoprimeverose, *Aspergillus oryzae*, xyloglucan, glycoside hydrolase

Isoprimeverose, α-D-xylopyranosyl-(1→6)-D-glucose, is a rare disaccharide produced during the enzymatic degradation of xyloglucan (XyG) [1]. XyG is present in cell walls and seeds of terrestrial plants. It is a major hemicellulosic polysaccharide that functions as a matrix polysaccharide. Some plants (e.g., *Tamarindus*) accumulate large amounts of XyG in their seeds as a storage polysaccharide [2]. Additionally, XyG is also used in foods as a viscosity-increasing polysaccharide. XyG has β-(1→4)-glucan as its main chain, and α-(1→6)-linked xylopyranosyl side chains are attached to glucopyranosyl residues of XyG main chain. The xylopyranosyl side chains are often modified with β-(1→2)-linked galactopyranosyl residues. In addition, the side chains of XyG can be modified with other saccharides such as fucose and arabinose [3]. *Aspergillus oryzae*, an important fungus used in traditional Japanese brewing, produces several types of glycoside hydrolases that degrade XyG [4]. *Aspergillus oryzae* degrades XyG into XyG oligosaccharides (XyG-oligos) using two different types of XyG specific endo-β-1,4-glucanases (xyloglucanase: EC 3.2.1.151): Xeg5A and Xeg12A (Fig. 1) [5]. XyG-oligos are then degraded into isoprimeverose, L-fucose, D-galactose, and D-glucose via the cooperative action of isoprimeverose-producing oligoxyloglucan hydrolase (IpeA, EC 3.2.1.120) [6], α-1,2-L-fucosidase (AfcA, EC 3.2.1.63) [7] and β-galactosidase (LacA, EC 3.2.1.23) [8]. The isoprimeverose-producing oligoxyloglucan hydrolase IpeA strictly recognizes and releases isoprimeverose units from the non-reducing ends of XyG-oligos [9, 10]. Isoprimeverose is then degraded into D-xylose and D-glucose by intracellular and extracellular α-xylosidases (AxyA and AxyB, respectively; EC 3.2.1.177) [11, 12]. In addition to AxyA and AxyB, *A. oryzae* has another intracellular α-xylosidase (AxyC), but its hydrolytic activity toward isoprimeverose is very low [13]. Expression of genes encoding glycoside hydrolases related to the degradation of XyG-oligos was induced in the presence of XyG-oligos [6, 8]. Other *Aspergillus* species, such as *A. nidulans* and *A. niger*, also produce XyG degradation-related enzymes

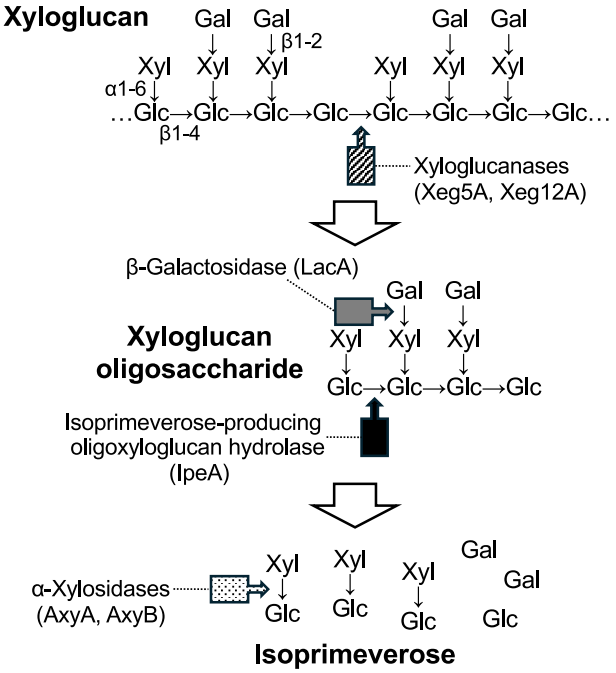

Fig. 1. XyG degradation-related glycoside hydrolases in *A. oryzae*. Glc, glucopyranosyl residue; Gal, galactopyranosyl residue; Xyl, xylopyranosyl residue.

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Abbreviations: XyG, xyloglucan; XyG-oligo, xyloglucan oligosaccharide; MM, minimal medium; AnCel, cellulase mixture from *A. niger*; XyG-AnCel, xyloglucan treated with *A. niger* cellulase; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; *p*NP, *p*-nitrophenyl.

[14]. Since isoprimeverose is a rare disaccharide, its functionality remains unclear. In this study, we developed a method for producing isoprimeverose from XyG using the culture supernatant of *A. oryzae*.

XyG (tamarind gum obtained from tamarind seeds) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). To remove impurities contained in the XyG, the XyG solution was treated with α-amylase and protease, followed by dialysis and freeze-drying. A cellulase mixture from *A. niger* (AnCel) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and low-molecular-weight compounds were removed using ultrafiltration [15]. To cultivate *A. oryzae*, XyG was partially decomposed by AnCel treatment to reduce its viscosity and produce trace amounts of XyG-oligos. AnCel (400 μg) was added to 70 mL of 1 % XyG and incubated at 40 ˚C until the viscosity of XyG had decreased to approximately 10 %. The viscosity was measured as described previously [16]. AnCel-treated XyG (XyG-AnCel) was boiled for 20 min to inactivate AnCel. The screening of *A. oryzae* strains that efficiently degrade XyG was carried out as follows: *A. oryzae* RIB40 (isolated from cereal, genome-sequenced reference strain), RIB128 (sake koji), RIB143 (sake koji), RIB163 (sake koji), RIB301 (shoyu koji), RIB915 (shoyu koji), RIB1108, and RIB1178 strains were cultured in 2 mL of minimal medium (MM, pH 6.5) containing 6 mg/mL sodium nitrate, 1.52 mg/mL potassium dihydrogen phosphate, 0.52 mg/mL potassium chloride, 0.52 mg/mL magnesium sulfate heptahydrate, trace elements, and 5 mg/mL XyG-AnCel at 30 ˚C for 3 days without shaking. A portion of the culture supernatant was collected daily, and XyG-AnCel degradation was confirmed by thin-layer chromatography (TLC) [7]. *Aspergillus oryzae* RIB915 strain showed much higher XyG-degrading activity than the other strains and produced low-molecular-weight XyG-oligos (Fig. 2).

The *A. oryzae* RIB915 strain was cultured in 20 mL of MM containing saccharide (5 mg/mL D-glucose, 5 mg/mL cellobiose, 5 mg/mL D-xylose, or 5 mg/mL XyG-AnCel) at 30 ˚C, 150 rpm for 3 days. *Aspergillus oryzae* cells were removed using a cell strainer (100 μm) and filtration (0.45 μm), and the glycoside hydrolase activities of culture supernatant were measured as follows: For chromogenic (*p*-nitrophenyl, *p*NP) substrates, 20 μL of reaction mixture containing 50 mM sodium acetate buffer (pH 5.0), 4 μL of culture supernatant, and 2 mM *p*NP-substrate (*p*NP β-D-glucopyranoside, *p*NP β-D-galactopyranoside, *p*NP β-D-xylopyranoside, or *p*NP α-D-xylopyranoside) were incubated at 40 ˚C for 30 min. After the reaction, 50 μL of 1 M sodium bicarbonate was added to the reaction mixture, and the concentration of released *p*-nitrophenol was determined by absorbance measurement at 405 nm. *Aspergillus oryzae* RIB915 cells showed β-glucosidase activity under all culture conditions, but β-glucosidase activity was relatively low in the MM containing XyG-AnCel (Fig. 3A). β-Galactosidase and β-xylosidase activities were highly induced in the MM containing XyG-AnCel, but not in other media (Fig. 3A). β-Galactosidase activity is important for the release of galactopyranosyl residues attached to xylopyranosyl side chains, which prevents the hydrolytic activity of the isoprimeverose-producing oligoxyloglucan hydrolase IpeA [6, 8]. The α-xylosidase activity toward *p*NP α-D-xylopyranoside was very low under all culture conditions (Fig. 3A), but please note that the α-xylosidase activities of AxyA and AxyB toward *p*NP α-D-xylopyranoside were much lower than those toward isoprimeverose and XyG-oligos [11, 12]. The hydrolytic activity of the culture supernatant toward XyG (Fig. 3B) was measured as follows: A 30 μL reaction mixture containing 50 mM sodium acetate buffer (pH 5.0), 5 μL of culture supernatant, and 5 mg/mL XyG was incubated at 40 ˚C for 30 min. The concentration of the reducing sugars produced was measured using the 3,5-dinitrosalicylic acid reagent method [17]. The XyG-hydrolysis activity, including both endo- (e.g., xyloglucanase) and exo- (e.g., β-galactosidase and isoprimeverose-producing oligoxyloglucan hydrolase) enzymes, of *A. oryzae* RIB915 was highly induced in the presence of XyG-AnCel but was not detected in other media containing D-glucose, cellobiose, or D-xylose as a carbon source (Fig. 3B).

The supernatant of *A. oryzae* RIB915 cells cultured in MM containing XyG-AnCel was concentrated using a Vivaspin Turbo 15 (10 K, PES) ultrafiltration unit (Sartorius Japan K.K., Tokyo, Japan), and low-molecular-weight compounds were removed. The protein concentration of the concentrated enzyme mixture in the culture supernatant of *A. oryzae* RIB915 cells (RIB915 MM-XyG-AnCel enzyme mixture) was measured using a NanoDrop Lite (Thermo Fisher Scientific Inc., Waltham, MA, USA). Next, we investigated whether isoprimeverose could be produced from XyG using the concentrated culture supernatant. The reaction mixture (100 μL) containing 8 mg/mL XyG, 50 mM sodium acetate buffer (pH 5.0), and 16 μg of RIB915 MM-XyG-AnCel enzyme mixture was incubated at 40 ˚C for 1−24 h, and the saccharides produced were analyzed by TLC (Fig. 3C) and a high-performance liquid chromatography (HPLC) system equipped with a refractive index detector and a TSKgel Amide-80 5-μm column (4.6 mm I.D. × 250 mm; Tosoh Corporation, Tokyo, Japan). XyG was completely degraded into mono- (D-galactose and D-glucose) and di- (isoprimeverose) saccharides within 24 h (Fig. 3C and 4A). These monosaccharides and isoprimeverose (Fig. 4A) were identical to those produced from XyG using purified recombinant

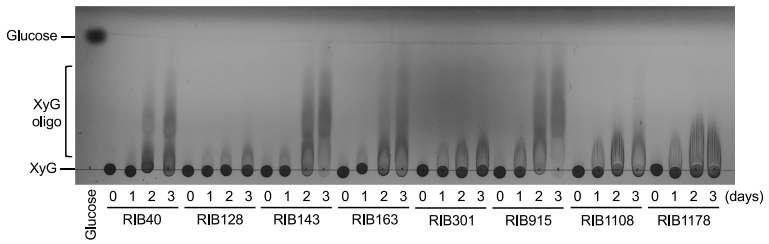

Fig. 2. Degradation of XyG by *A. oryzae* RIB strains.
The culture supernatants of *A. oryzae* cells were analyzed using TLC.

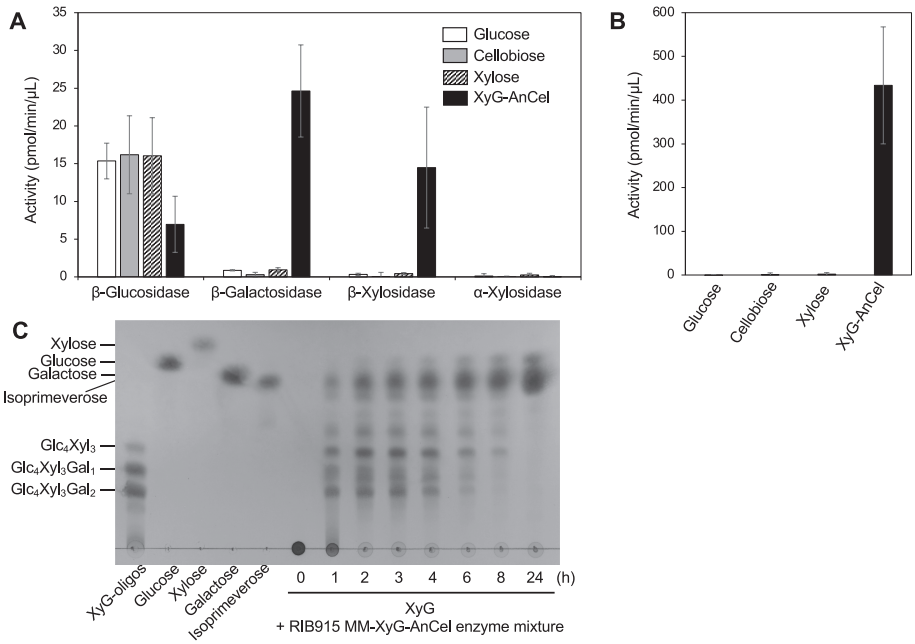

Fig. 3. Glycoside hydrolase activities of culture supernatants of *A. oryzae* RIB915 strain.

(A) β-Glucosidase, β-galactosidase, β-xylosidase, and α-xylosidase activities of culture supernatant of *A. oryzae* RIB915 cells cultured in MM containing 0.5 % D-glucose (white bars), 0.5 % cellobiose (gray bars), 0.5 % D-xylose (slashed bars), or 0.5 % XyG-AnCel (black bars). (B) Glycoside hydrolase activity toward XyG in the culture supernatant of *A. oryzae* RIB915 cells cultured in MM containing 0.5 % D-glucose, 0.5 % cellobiose, 0.5 % D-xylose, or 0.5 % XyG-AnCel. Error bars indicate standard deviation (n = 3) of independent cultivations. (C) TLC analysis of time-dependent changes in degradation products produced from XyG using concentrated culture supernatant of *A. oryzae* RIB915 cells cultured in MM containing 0.5 % XyG-AnCel.

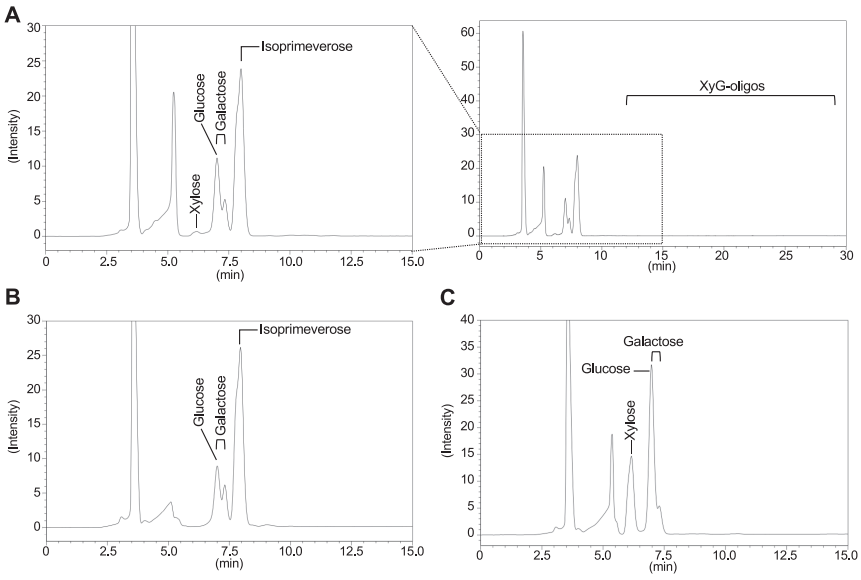

Fig. 4. (A) HPLC analysis of mono-, di-, and oligosaccharides produced from XyG using RIB915 MM-XyG-AnCel enzyme mixture (24-h incubation). (B) XyG was degraded by purified recombinant Xeg12A, IpeA, and LacA. (C) Mono- and disaccharides produced from XyG using RIB915 MM-XyG-AnCel enzyme mixture were treated with purified recombinant α-xylosidase AxyB.

xyloglucanase Xeg12A [5], isoprimeverose-producing oligoxyloglucan hydrolase IpeA [6], and β-galactosidase LacA [8] (Fig. 4B). After 24-h incubation, the concentrations of isoprimeverose and D-xylose produced from XyG (8 mg/mL) by RIB915 MM-XyG-AnCel enzyme mixture were 4.64 ± 0.06 mg/mL and 0.154 ± 0.003 mg/mL, respectively. These results indicated that approximately 0.6 mg of isoprimeverose was produced from 1 mg of XyG and produced isoprimeverose hardly degraded into D-xylose and D-glucose by α-xylosidase. The produced isoprimeverose (Fig. 4A) was completely degraded into D-glucose and D-xylose by the addition of purified recombinant α-xylosidase AxyB [12] (Fig. 4C). Previously, Oka et al. reported that the expression of *axyB* gene is regulated by the transcription factor XlnR, which activates the expression of genes encoding xylanolytic and cellulolytic enzymes [18]. It is speculated that the induced

expression of *axyB* causes a decrease in the productivity of isoprimeverose.

Previously, the production of isoprimeverose by heterologous expression and purification of bacterial isoprimeverose-producing oligoxyloglucan hydrolase was reported [19]. The method developed in this study enabled the production of large amounts of isoprimeverose from XyG without any genetically modified microorganisms or purification of enzymes. In future studies, we aim to investigate the physiological functions and prebiotic effects of isoprimeverose.

CONFLICTS OF INTEREST

The authors declare no conflict of interests.

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