Electronic Supplementary Information (ESI)

Novel β-galactosynthase - β-mannosynthase dual activity of β-galactosidase from *Aspergillus oryzae* uncovered using monomer sugar substrates

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I. Materials and Methods

Materials

The β-galactosidase from *Aspergillus oryzae* (EC 3.2.1.23) used in this study is a powdered form (11.2 units/mg solid) and was purchased from Sigma Chemical Co.. Ultrafiltration membranes were supplied from Milipore, Carrigtwohill, Co. Cork, Ireland. Phosphate buffer was prepared from 0.05 M KH_2PO_4, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 0.1 M NaOH was used to adjust the pH. D-Galactose, D-Mannose, 2,5-dihydroxybenzoic acid (DHB), 2,5-dihydroxybenzoic acid Sodium salt (DHBNa) and *O*-benzylhydroxylamine hydrochloride (BOA) were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Glycoblotting beads (BlotglycoH™) was purchased from Sumitomo Bakelite, Co. (Tokyo, Japan). *Tri-N*-acetylchitotriose was purchased from SEIKAGAKU CORPORATION (Tokyo, Japan). Unless otherwise noted, solvents and other reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI), Tokyo Chemical Industry (Tokyo, Japan), and Wako Pure Chemical Industries Ltd. (Tokyo, Japan). MALDI-TOF/MS analysis was performed using Ultrflex I (Bruker Daltonics, Bremen, Germany) in a reflector, positive ion mode, typically totalling 200 x 15 shoots, controlled, and analyzed by the Flexcontrol 3.0 software package (Bruker Daltonics, Bremen, Germany) according to the protocol previously reported (55-56). The Proton NMR was recorded using Varian UnityInova 500 MHz (Agilent Inc., USA). ^{1}H-^{13}C and ^{1}H-^{1}H correlations NMR were recorded using 600 MHz Bruker.

II. Methods

Enzyme purification

The crude *Ao*- β-gal enzyme (1.0 mg) was dissolved completely in 1.0 mL of mili Q water and 500 µL was loaded to each ultrafiltration membrane with nominal molecular weight limit of 30,000 (YM-30). The solutions were centrifuged at 13,000 g and 4 ^{o}C for 10 minutes, added with 400 µL of mili Q water and re-centrifuged; the process was repeated until all the extenders were removed. A 1.0 µL enzyme concentrate was analyzed using MALDI-TOF to verify the removal of the extender.

Determination of Transglycosylation Activity of *Ao*- β-gal

Free D-galactose and D-mannose monosaccharides (200 mg) were each dissolved in buffer (50 mM KH_2PO_4, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol; pH 4.7) and added with the purified *Ao*- β-gal (100 U) to a total volume of 1.0 mL. The mixtures were incubated for 24 hours at 40 ^{o}C and 45 ^{o}C for galactose and mannose, respectively. A 10 µL solution was lyophilized and the solid was dissolved in miliQ water to make 5 µg/µL solution and the samples were enriched using glycoblotting analysis and analyzed using Ultrflex I MALDI-TOF/MS to confirm the presence of products.

Glycoblotting Enrichment of Saccharides in Sample Mixture[15]

The summary of enrichment process was shown in Figure 1. Briefly, a 100 µL aliquot of BlotglycoH beads in a 10 mg/mL suspension in water was placed into a MultiScreen Solvinert filter plate well (Milipore, Billerica, MA) and the water was drained by a vacuum. A 10 µL of 5 µg/µL sample solution and 10 µL of 0.225 mM of *tri-N*-acetylchitotriose, as the internal standard, were impregnated into the well followed by addition of 180 µL of 2% acetic acid in acetonitrile. The plate was incubated at 80 ^{o}C for 45 minutes to dryness in a thermostat to capture the whole saccharides in the sample mixture onto the beads. The plate was washed twice with 200 µL of miliQ water and 1% triethylamine in methanol,

added with 100 µL of 10% acetic acid in methanol and incubated at 25 ^{o}C for 30 minutes to cap the unreacted hydrazide functional groups on the beads. After incubation, the solution was removed by vacuum and then washed twice successively with 200 µL of 10 mM HCl, methanol, and dioxane. Trans-iminization reaction on the oligosaccharides captured on beads was performed by addition of 20 µL of 50 mM BOA followed by treatment with 180 µL of 2% acetic acid in acetonitrile. The plate was incubated for 45 minutes at 80 ^{o}C. The BOA-tagged saccharides were eluted with 100 µL of water and analyzed using Ultraflex I MALDI-TOF/MS.

***MALDI-TOF/MS* Analysis**

The recovered BOA-tagged saccharides (1.0 µL) was mixed with the same volume of the matrix DHBNa:DHB (1:9) consisting 10mg/mL in 30% acetonitrile on a target plate MYP 384 (polished steel TF, Bruker Daltonics, Bremen, Germany) and dried to afford crystals of the sample-matrix. The crystals were analyzed using MALDI-TOF/MS. For quantification, the analysis was performed in three trials at 5000 laser shots per sample and the data were reported as the average (±SEM) of the trials.

Product Isolation and Purification

The remaining mixtures containing the *Ao*- β-gal and monosaccharides were deactivated in a heatblock at 90 ^{o}C for 20 minutes and centrifuged at 100, 000 rpm for 30 minutes. The supernatants were then lyophilized and acetylated. In brief, the lyophilized supernatants were added with anhydrous sodium acetate and dissolved in acetic anhydride. The solutions were stirred overnight at 80 ^{o}C in an oil bath, cooled at room temperature, pounded in cold saturated sodium bicarbonate solution and stirred for 1.5 hours. The solutions were extracted twice with equal volumes of CH_2Cl_2. The combined CH_2Cl_2 layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The concentrated sample was subjected to flash column chromatography using ethyl acetate and hexane (2:1) as eluants and dried under reduced pressure to isolate the peracetylated disaccharide

products. The isolated peracetylated disaccharides were then completely dried overnight in a vacuum and structurally characterized using 1D and 2D NMR.

Product Characterization

The isolated peracetylated disaccharide products were dissolved in deuterated chloroform (Chloroform-*d*) and were transferred into NMR sample tubes. The samples were characterized using Varian UnityInova 500 MHz (Agilent Inc., USA) for ^{1}H-NMR analysis and using 600 MHz Bruker for ^{1}H-^{13}C and ^{1}H-^{1}H correlation spectroscopies.

Peracetylated Galactobiose Structural Characterization

2,3,4,6-tetra-*O*-Acetyl-β-D-galactopyranosyl-(1-6)-1,2,3,4-tetra-*O*-Acetyl-D-galactopyranose ($C_{28}H_{38}O_{19}$). Exact mass: 678.20 Da.

^{1}H NMR (500 MHz, CHLOROFORM-*d*) δ ppm 3.76 - 3.80 (m, 2 H) 3.88 (d, *J* = 1.11 Hz, 1 H) 3.99 (d, *J* = 1.04 Hz, 1 H) 4.12 - 4.16 (m, 2 H) 4.51 (d, *J* = 7.94 Hz, 1 H) 4.99 (d, *J* = 3.49 Hz, 1 H) 5.08 (d, *J* = 3.41 Hz, 1 H) 5.15 (d, *J* = 2.52 Hz, 1 H) 5.31 - 5.32 (m, 1 H) 5.37 (d, *J* = 1.11 Hz, 1 H) 5.42 (d, *J* = 0.97 Hz, 1 H) 5.68 (d, *J* = 8.34 Hz, 1 H).

Peracetylated Mannobiose Structural Characterization

2,3,4,6-tetra-*O*-Acetyl-β-D-mannopyranosyl-(1-6)-1,2,3,4-tetra-*O*-Acetyl-D-mannopyranose ($C_{28}H_{38}O_{19}$). Exact mass: 678.20 Da.

^{1}H NMR (500 MHz, CHLOROFORM-*d*) δ ppm 3.62 (dd, *J* = 11.18, 2.93 Hz, 1 H) 3.76 (dd, *J* = 11.18, 4.75 Hz, 1 H) 3.95 - 4.00 (m, 1 H) 4.01 - 4.05 (m, 1 H) 4.07 - 4.11 (m, 1 H) 4.27 (d, *J* = 4.91 Hz, 1 H) 4.30 (d, *J* = 4.94 Hz, 1 H) 4.83 (d, *J* = 1.46 Hz, 1 H) 5.26 (dd, *J* = 2.90, 2.04 Hz, 1 H) 5.28 (dd, *J* = 2.77, 1.85 Hz, 1 H) 5.30 - 5.31 (m, 1 H) 5.36 (d, *J* = 1.53 Hz, 1 H) 5.36 - 5.37 (m, 1 H) 6.05 (d, *J* = 1.92 Hz, 1 H)

III. Supplementary Figures

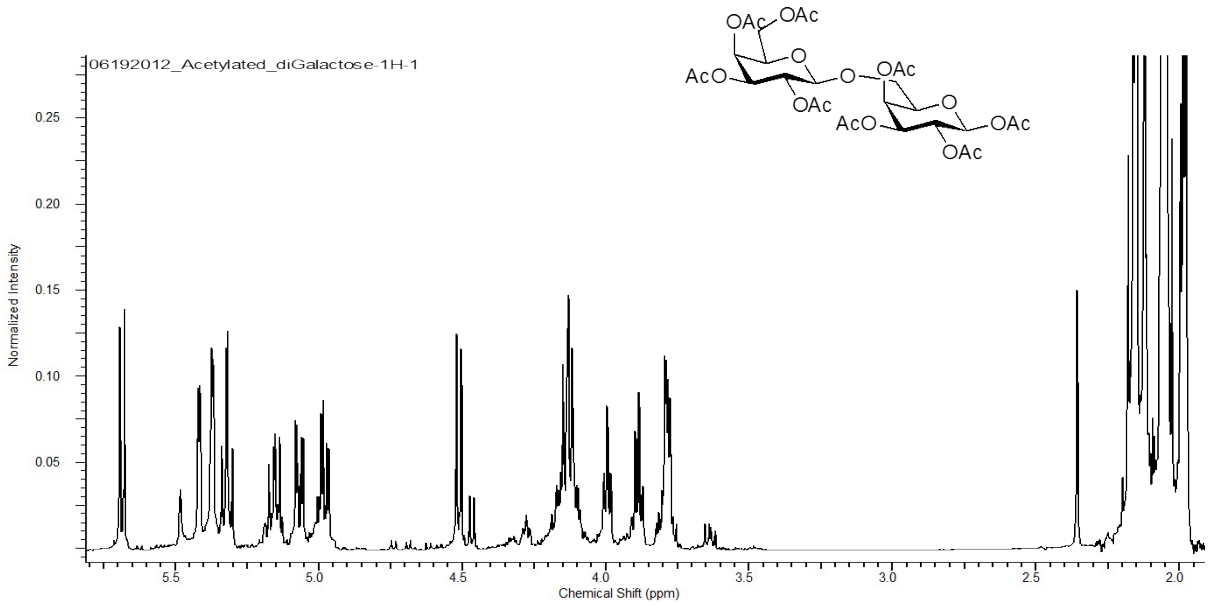

Figure S1. [1]H NMR Spectra of Peracetylated Galactobiose.

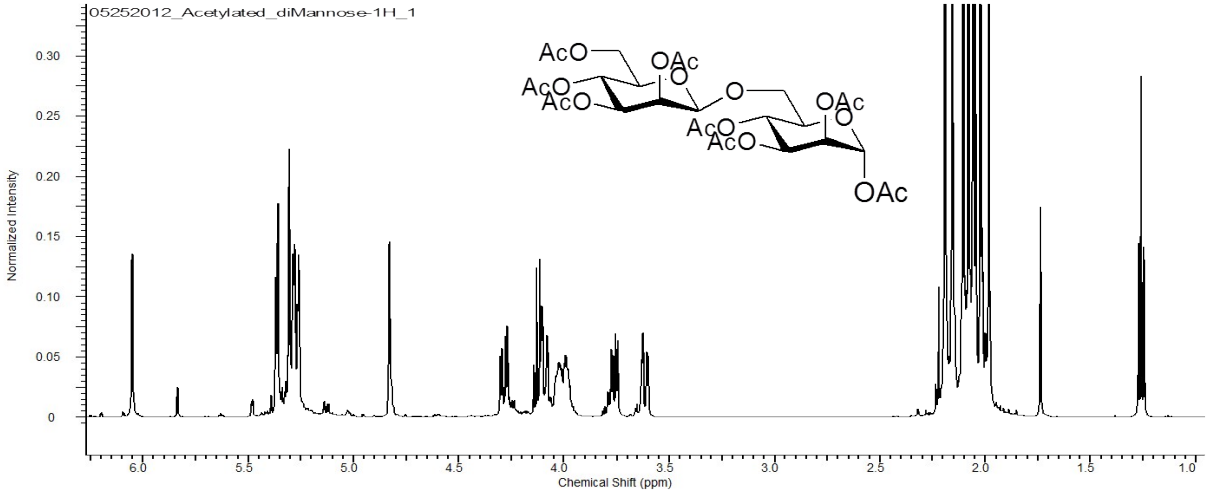

Figure S2. [1]H NMR Spectra of Peracetylated Mannobiose.