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Action of transglucosidase from *Aspergillus niger* on maltoheptaose and [U–^{13}C]maltose

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ABSTRACT

Oligosaccharides synthesized from a mixture of maltoheptaose and [U–^{13}C]maltose with transglucosidase [EC 2.4.1.24] from *Aspergillus niger* were investigated. When the reaction mixture was incubated at 15 °C for 1 h, several types of oligosaccharides with DP (degree of polymerization) 2 to DP8 containing α-D-Glc*p*-(1→6)-maltoheptaose were detected by liquid chromatography–mass spectrometry (LC–MS) and methylation analysis. Most of these compounds consisted of α-(1→4) linkages in the main chain and α-(1→6) linkages at the non-reducing ends. However, when the reaction mixture was incubated for 96 h, most of these products were converted into oligosaccharides with DP2 to DP5 consisting of only α-(1→6) linkages. These results suggested that *A. niger* transglucosidase rapidly transferred glucosyl residues to maltooligosaccharides, and gradually hydrolyzed both α-(1→4) linkages and α-(1→6) linkages at the non-reducing end, and transformed these into smaller molecules of mainly α-(1→6) linkages.

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1. Introduction

Transglucosidases [EC 2.4.1.24] catalyze transfer-reactions as well as hydrolysis in maltooligosaccharides.[1,2] Transglucosidases are widely distributed in microorganisms, plants, insects, and mammals, and some of them have been applied for the synthesis of oligosaccharides[3–9] and glycosylated compounds.[10–12] These enzymes from different sources show diverse substrate specificity and regioselectivity. *Bacillus stearothermophilus* α-glucosidase synthesizes maltotriose from maltose, whereas Brewer's yeast α-glucosidase synthesizes oligosaccharides consisting of α-(1→3), α-(1→4), and α-(1→6) linkages.[3] *Bifidobacterium adolescentis* α-glucosidase AglA synthesizes oligosaccharides from sucrose and trehalose, whereas another α-glucosidase AglB synthesizes oligosaccharides from maltose, sucrose, and melizitose.[4] *A. niger* transglucosidase synthesizes mainly isomaltose and panose, and small amounts of nigerose and kojibiose from maltose.[13–15] Digestion of long oligosaccharides such as maltotetraose and maltoheptaose with *A. niger* transglucosidase was investigated.[16] Several synthetic products had higher molecular weights than substrates; however, the structures of these products had not been determined. It remains to be clarified whether *A. niger* transglucosidase could transfer D-glucose residues from a short oligomer, such as maltose, to a long oligomer, such as maltoheptaose. For the determination of the ratio of α-(1→4) and α-(1→6) linkages of the synthetic oligomers larger than DP3, methylation analysis is more suitable than nuclear magnetic resonance (NMR), because NMR is accompanied with the heavy overlaps of proton resonances due to an inherent low dispersion of their chemical shifts, which results in difficulties of signal identification. Methylation analysis is widely used for the determination of mode of linkages between component monosaccharide residues in oligo- and poly-saccharides, and for the estimation of the ratio of glucose at non-reducing end.[17–19] [U–^{13}C]maltose is a useful substrate for obtaining information on [U–^{13}C]glucosyl residues transferred to the oligosaccharides, and also on the positions of their linkages. This paper reports the digestion pattern of *A. niger* transglucosidase in a mixture of maltoheptaose and [U–^{13}C]maltose.

2. Results and discussion

2.1. LC–MS analysis of transglucosidation digestion of maltoheptaose and [U–^{13}C]maltose with *A. niger* transglucosidase

The time course of *A. niger* transglucosidase reaction with a mixture of maltoheptaose and [U–^{13}C]maltose was examined by a size exclusion chromatography–evaporative laser scattering detector (SEC–ELSD, Fig. 1) and a liquid chromatography–mass spectrometry (LC–MS, Fig. 2). Maltoheptaose was consumed and several

Abbreviations: DP, degree of polymerization; ESI, electro-spray ionization; ELSD, evaporative laser scattering detector; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; PMAA, partially methylated alditol acetates; RP, reversed-phase; SEC, size exclusion chromatography; SIM, selected ion monitor; TIC, total ion chromatogram.

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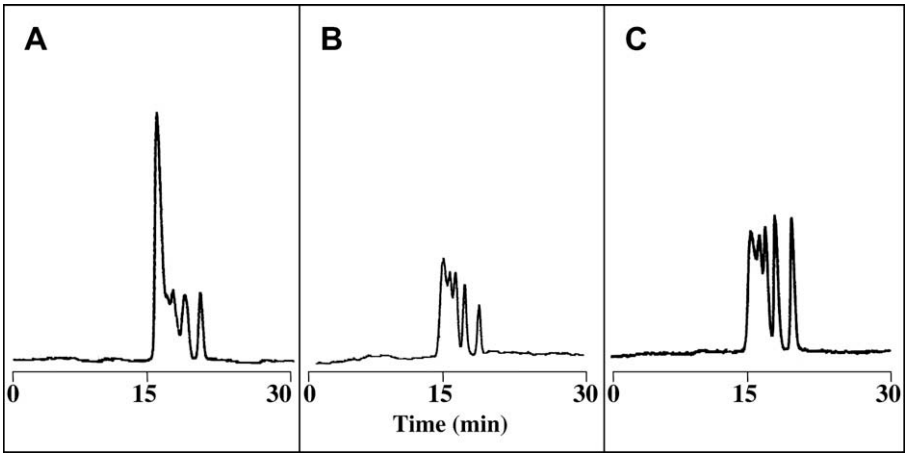


Figure 1. SEC–ELSD analysis of digests of maltoheptaose and [U–^{13}C]maltose with transglucosidase (column: TSK-gel G-Oligo-PW, 7.8 × 300 mm, 7 μm; flow rate: 0.5 mL/min; solvent: water): (A) digestion for 1 h, (B) digestion for 24 h, and (C) digestion for 96 h.

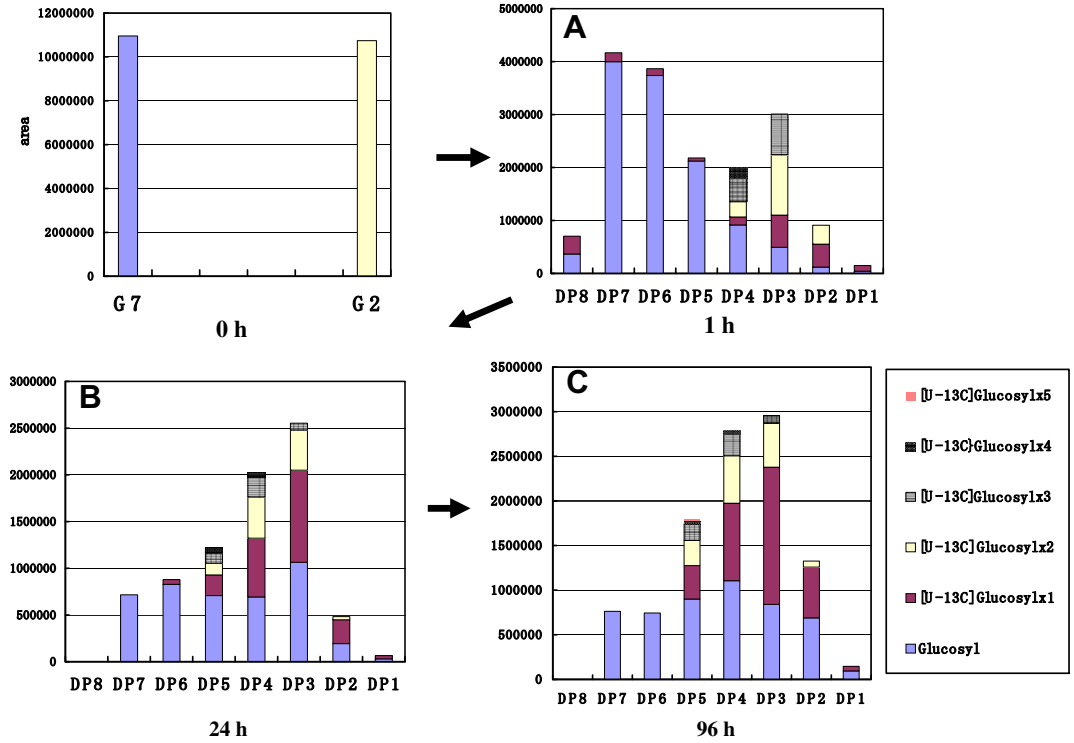


Figure 2. Molar ratios of [U–^{13}C]glucosyl residue-containing oligosaccharides during the digestion of maltoheptaose and [U–^{13}C]maltose with transglucosidase, as estimated from the peak areas of the combination of their molecular ion $[M–H]^-$ and formate cluster ion $[M+(HCOO)]^-$ from LC–MS analyses.

low-molecular compounds appeared within 96 h (Fig. 1). LC–MS revealed that the oligosaccharides with DP2-4 contained large amounts of [U–^{13}C]glucosyl residues, whereas oligomers with DP5-7 contained small amounts of [U–^{13}C]glucosyl residues, and oligomer with DP8 contained a [U–^{13}C]glucosyl residue at 1 h digestion (Fig. 2A). This result suggested that the enzyme transferred [U–^{13}C]glucosyl residues to all maltooligosaccharides with DP1-7, and the enzyme preferred the short chains with DP1-3 as substrates to synthesize DP2-4 (Fig. 2A). Because of the slow hydrolysis of maltoheptaose, the enzyme could transfer a [U–^{13}C]glucosyl residue to maltoheptaose in the initial stage to form an oligomer with DP8, which was not hydrolyzed rapidly with the enzyme. At 24 h digestion, the ratio of [U–^{13}C]glucosyl residues of the oligomers with DP5 increased (Fig. 2B). The oligomers with DP6-8 decreased at 96 h digestion, while the oligomers with DP2-5 increased, this result suggests that the transglucosidation from the long oligomers to the shorter oligomers continued (Fig. 2C).

2.2. Methylation analysis of products in the digestion of maltoheptaose and [U–^{13}C]maltose with *A. niger* transglucosidase

The reaction of maltoheptaose and [U–^{13}C]maltose with the transglucosidase was performed, and the resulting oligosaccharides were obtained by RP-HPLC (Fig. 3) and were subjected to methylation analysis (Fig. 4) and LC–MS. Probable structures of the main products based on these analyses are listed in Table 1. Methylation analyses revealed that oligosaccharides with DP3 (A-2) to DP8 (A-7) contained more α-(1→4) linkages than α-(1→6)

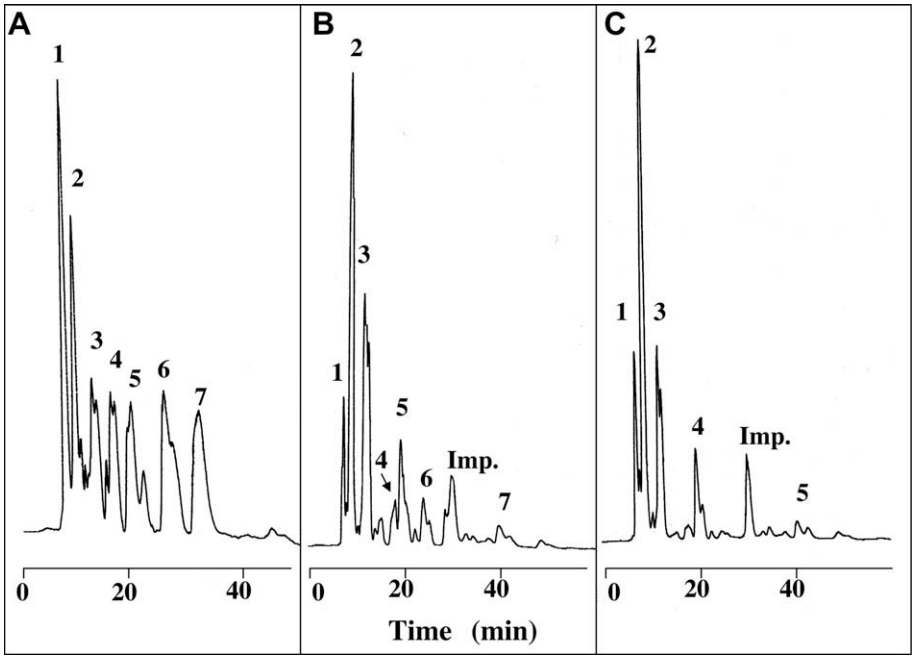


Figure 3. Preparative RP-HPLC of SEC-purified samples from the digests of maltoheptaose and [U–^{13}C]maltose with transglucosidase (column: Hydrosphere C18, HS303, 4.6 × 250 mm, 5 μm; flow rate: 0.6 mL/min; solvent: water:CH_3CN (95:5 v/v)): digestion for (A) 1 h, (B) 24 h, and (C) 96 h.

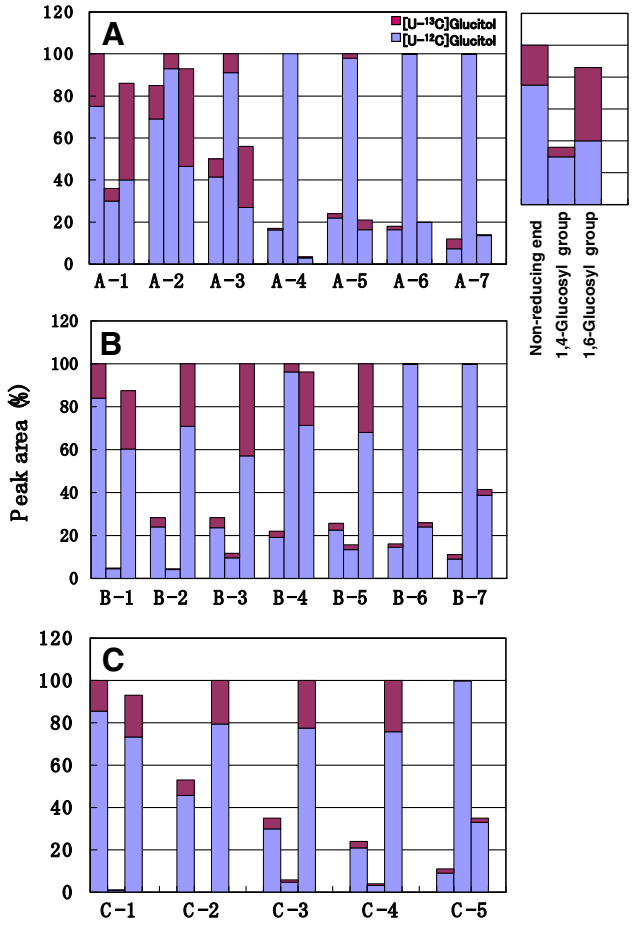


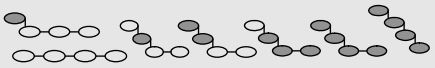
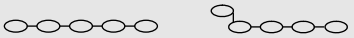
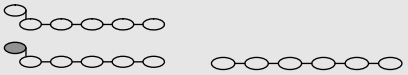
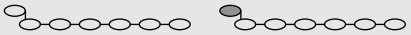
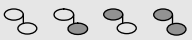
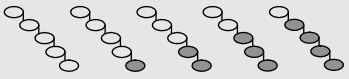
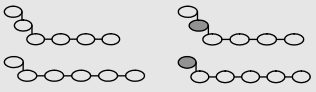
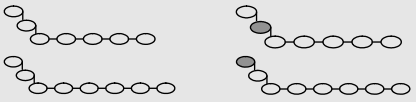
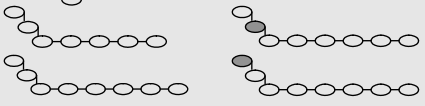
Figure 4. Methylation analysis of fractions obtained by preparative RP-HPLC from the digests of maltoheptaose and [U–^{13}C]maltose with transglucosidase as shown in Figure 3: digestion for (A) 1 h, (B) 24 h, and (C) 96 h.

linkages when the reaction was performed for 1 h (Fig. 4A), suggesting that these oligomers consisted of α-(1→4) linkages in the main chain and α-(1→6) linkage(s) at the non-reducing ends. The ratio of [U–^{13}C]glucosyl residues at the non-reducing end in A-7 was 39%, which was higher than that in A-5 or A-6 (Fig. 4A). This result supported the present speculation that the enzyme transferred [U–^{13}C]glucosyl residues to the non-reducing end of maltoheptaose prior to the generation of A-5 and A-6. Patur et al. reported that several products from the maltoheptaose digestion for 4 h were hydrolyzed by β-amylase.[16] Thus, there could also be maltooligosaccharides consisting of only α-(1→4)-glucosyl residues in oligomers A-2 (DP3) and A-3 (DP4) as shown in Table 1. The oligomers B-1 (DP2) to B-4 (DP5) from the digestion for 24 h contained more α-(1→6) linkages than α-(1→4) linkages (Fig. 4B). After 96 h, the synthetic oligomers C-1 (DP2) to C-4 (DP5) consisted of mainly α-(1→6) linkages (Fig. 4C). On the other hand, C-5 (DP7, DP8) contained both α-(1→4) linkages and α-(1→6) linkages in the ratio of 2:1, this result suggests that the enzyme transferred another α-(1→6)-linked D-glucosyl residue to the non-reducing ends of A-5 or A-6 (Table 1).

The digestion of maltoheptaose without [U–^{13}C]maltose was examined with the enzyme using the same procedure, and the resulting oligomers were obtained by HPLC. LC–MS and methylation analyses (Fig. 5) gave equivalent results as those obtained from the reaction mixture of maltoheptaose and [U–^{13}C]maltose. In addition, a small amount of product (D-8) was obtained and the ratio of the α-(1→6) linkage was about twice as much as that of D-7 (DP8). This result indicated that D-8 was α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-maltoheptaose (DP9).

Taking these results into consideration, we postulated a simple reaction pathway. Firstly, *A. niger* transglucosidase transferred a [U–^{13}C]glucosyl residue to the non-reducing end of maltoheptaose, while the enzyme degraded maltoheptaose into maltooligosaccharides with DP1-6. The enzyme then transferred D-glucosyl residues to the oligomers with various DP, mainly DP2-4. As the digestion proceeded, the enzyme slowly transferred the α-(1→6)-linked D-glucosyl residues of the long oligomers with DP5-8 to the short

Table 1
Molecular ion from LC–MS, DP, and probable structure of the main products: digestion for (A) 1 h, (B) 24 h, and (C) 96 h

Fr	MS $[M-H]^-$(m/z)	DP	Probable main products
A-1	341.2,347.2,353.2	2	
A-2	503.3, 509.3, 515.4, 521.3	3	
A-3	665.3,671.5,677.4, 683.5, 689.5	4	
A-4	827.4	5	
A-5	989.5,995.5	6	
A-6	1151.5, 1152.7, 1157.4, 1158.4	7	
A-7	1313.6,1314.6, 1319.6, 1320.6	8	
B-1	341.2,347.2, 353.2	2	
B-2	503.2,509.1,515.3	3	
B-3	665.2,671.3, 677.3, 683.4, 689.6	4	
B-4	828.4,833.4, 839.6, 845.5	5	
B-5	827.4,833.5, 839.5, 845.5, 851.4	5	
B-6	989.5,995.4	6	
B-7	1151.4,1152.4,1157.4, 1158.4,1313.6,1314.6, 1319.6,1320.6	7,8	
C-1	341.2, 347.2	2	
C-2	503.2,509.1,515.2	3	
C-3	665.4,671.4,677.5	4	
C-4	827.4, 833.5,839.5, 845.4, 851.5	5	
C-5	1151.4,1152.4 1157.4,1313.6, 1319.6,	7,8	

: Glucose, : [U-^{3}C]glucose : 1,4-linkage, : 1,6-linkage.

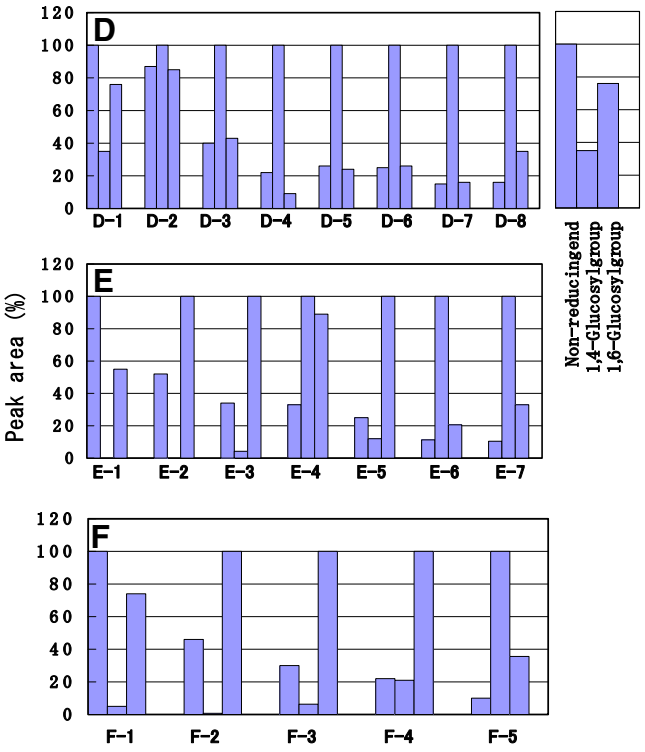


Figure 5. Methylation analysis of fractions obtained by preparative RP-HPLC from the digests of maltoheptaose with transglucosidase: digestion for (D) 1 h, (E) 24 h, and (F) 96 h.

oligomers with DP1-4 to accumulate isomaltooligosaccharides with DP2-5.

In the present experiments, *A. niger* transglucosidase converted [U–^{13}C]maltose and maltoheptaose into the products with DP2-8 with a yield of 25% after 24 h. *Aspergillus nidulans* α-glucosidase (AgdB) had strong transglucosidation activity to convert maltose into isomaltose, panose, and isomaltotriose with a yield of 50%.[20] This enzyme hydrolyzed maltotriose effectively; however, oligomers longer than DP4 were less favored. *Aspergillus awamori* α-glucosidase also synthesized isomaltose, panose, and isomaltotriose from maltose; however, no oligomer longer than DP4 was synthesized.[21] We therefore assumed that *A. nidulans* AgdB and *A. awamori* α-glucosidase could synthesize isomaltooligosaccharides around DP3 in the present system. On the other hand, *Aspergillus oryzae* transglucosidase synthesized oligomers with DP2-5 (51% yield) and isomaltooligosaccharide with DP6 (0.14% yield) from maltose.[22] Thus, *A. oryzae* transglucosidase might synthesize isomaltooligosaccharides up to DP6 in the present system.

3. Experimental

3.1. Materials

3.1.1. Chemicals

[U–^{13}C]Maltose was purchased from Omicron Co. (USA). Maltoheptaose was purchased from Tokyo Kasei Co. (Japan). Maltohexaose, maltopentaose, maltotetraose, and maltotriose were purchased from Wako Pure Chemicals Co. (Japan). All the chemicals were used without further purification.

3.1.2. Enzyme

Transglucosidase (from *A. niger*, 'Transglucosidase L') was a gift from Amano Enzyme Co. (Japan) and was purified to be free from glucoamylase as described below.

3.2. Methods

3.2.1. Purification of transglucosidase

The crude transglucosidase (5 g) was dissolved in 25 mL of 0.02 M phosphate buffer (pH 7.2) and was dialyzed against the same buffer. The enzyme solution was subjected to DEAE-Sepharose Fast Flow column chromatography (volume 500 mL; GE Healthcare Bio-Sciences Co., USA) equilibrated with 0.02 M phosphate buffer (pH 7.2) by monitoring at 280 nm. The enzyme was eluted with a NaCl (0–1 M) linear gradient. The active fractions were collected and concentrated by ultrafiltration (Hydrosart 10-kD cutoff, Sartorius AG, Germany). The enzyme activity was measured by the method reported by Tanimura et al.[23] with slight modifications. The enzyme solution (0.5 mL) was mixed with 0.5 mL of 7.5 mM *p*-nitrophenyl-glucoside in 10 mM sodium acetate buffer (pH 5.0). After incubation for 10 min at 40 °C, the mixture was cooled on ice, and 0.5 mL of 10% Na_2CO_3 was added. The amount of *p*-nitrophenol released was measured using a UV detector at 420 nm. One unit of transglucosidase activity was defined as the amount of enzyme that hydrolyzed 1 μmol of *p*-nitrophenyl-glucoside per min under the above conditions. The final concentration of the enzyme solution was approximately 60 unit/mL, and the enzyme solution was used for experiments.

3.2.2. LC–ESI MS analysis

LC electro-spray ionization (ESI) MS analysis was performed on an HP1100 (Hewlett–Packard) HPLC system coupled to a Navigator (Thermoquest) quadrupole mass spectrometer equipped with an ESI interface. The column (Capcell pak, NH_2, 5 μm, 2.0 × 250 mm, Shiseido, Japan) was used with a flow rate of 0.1 mL/min and had an injection volume of 30 μL. Solvent A: CH_3CN; solvent B: 0.05 mol/L ammonium formate buffer, pH 8; A:B 70:30–50:50 (25 min)–50:50 (35 min)–70:30 (40 min). Samples were dissolved in water. ESI capillary voltage: 3.5 keV; cone voltage: 35 V; negative mode.

3.2.3. GC–MS analysis

GC–MS analysis was performed on an HP5890 (Hewlett–Packard) gas chromatograph coupled to an HP5972 (Hewlett–Packard) mass spectrometer using an NB-1 column (0.25 mm × 60 m × 0.4 μm, GL Sciences, Inc., Japan). After sample injection in split mode, the oven temperature program was started and the temperature was maintained for 2 min at 140 °C, raised at 2 °C/min from 140 to 200 °C, then at 10 °C/min from 200 to 250 °C, and then maintained for 18 min at 250 °C. Mass spectra in the electron impact (EI) mode were generated at 70 eV, with the ion source temperature at 220 °C, and at a scan range from *m/z* 40 to 550.

3.2.4. Analytical and preparative SEC–ELSD

The analytical and preparative HPLC system (Waters Co., USA) consisted of a 600S gradient pump system combined with an ELSD 2000 (Alltech, USA) and an SEC column (TSK-gel G-Oligo-PW, 7.8 × 300 mm, 7 μm, Tosoh, Japan); flow rate, 0.5 mL/min; solvent, water. A mixture of maltoheptaose, maltotriose, maltose, and glucose was used as marker.

3.2.5. Preparative RP-HPLC

The HPLC system (Waters Co. USA) consisted of a 600S gradient pump system combined with an ELSD and an RP column (Hydrosphere C18, HS303, 4.6 × 250 mm, 5 μm, YMC Co., Ltd, Japan); flow rate, 0.6 mL/min; solvent, water–CH_3CN (95:5 v/v).

3.2.6. LC–MS analysis of transglucosidase action on a mixture of maltoheptaose and [U–^{13}C]maltose

Maltoheptaose (5.0 mg, 4.3 μmol) and [U–^{13}C]maltose (1.5 mg, 4.2 μmol) were dissolved in 16 μL of water. To the solution, 1 μL of transglucosidase solution (0.06 U) was added, the mixture was

stirred at 15 °C, and then 3-μL aliquots of the reaction mixture were withdrawn at different time intervals. The aliquots were mixed with 0.1 mL of boiled water immediately, heated for 5 min at 100 °C, and then cooled to room temperature. Next, 30 μL of the mixture was subjected to LC–MS, and 25 μL of the mixture was subjected to analytical SEC column chromatography. The molar ratios of [U–^{13}C]glucosyl residue-containing oligosaccharides were estimated from the peak area of the combination of their molecular ion $[M–H]^-$ and formate cluster ion $[M+(HCOO)]^-$ of LC–MS. Molar ratios of the detected oligosaccharides (from DP1 to DP8) were estimated by determining the relative peak area of an equimolar mixture of glucose (DP1), maltose (DP2), maltotriose (DP3), maltotetraose (DP4), maltopentaose (DP5), maltohexaose (DP6), and maltoheptaose (DP7) detected by LC–MS. Maltooctaose (DP8) was not available commercially, so the molar-response of this oligosaccharide (DP8) was estimated as being the same as that of oligosaccharide DP7. The relative peak areas of equimolar amounts of these oligosaccharides were determined as follows:

Peak ratios of DP1: DP2: DP3: DP4: DP5: DP6: DP7: DP8 were 0.6: 1: 0.82: 0.65: 0.29: 0.12: 0.04: 0.04.

Response-factors were as follows:

DP 1 (1.67), DP 2 (1), DP 3 (1.22), DP 4 (1.54), DP 5 (3.45), DP 6 (8.33), DP 7 (25), and DP 8 (25).

3.2.7. LC–MS analysis of transglucosidase action on maltoheptaose

The reaction was performed as described above, except that [U–^{13}C]maltose was omitted.

3.2.8. Preparation of oligosaccharides from the mixture of maltoheptaose and [U–^{13}C]maltose synthesized with transglucosidase

Maltoheptaose (43 mg, 0.037 mmol) and [U–^{13}C]maltose (13 mg, 0.036 mmol) were dissolved in 0.14 mL of water. To the solution, 10 μL of transglucosidase solution (0.5 U) was added, and the mixture was stirred at 15 °C. Reactions were performed for various lengths of time (1, 24, and 96 h), and 1 mL of boiling water was added to the mixture after each reaction, and these reaction mixtures were heated at 100 °C for 10 min. Next, each mixture was cooled to room temperature and 0.1 mL of the mixture was subjected to SEC (column: TSK-gel G-Oligo-PW, 7.8 × 300 mm, 7 μm; flow rate: 0.5 mL/min; solvent: water). This procedure was repeated ten times. Each fraction was combined and lyophilized. Fraction 1 with t_R 16.0–19.7 min (for 1 h, 19.9 mg; for 24 h, 13.9 mg; and for 96 h, 8.4 mg), fraction 2 with t_R 19.7–21.5 min (for 1 h, 8.2 mg; for 24 h, 5.9 mg; and for 96 h, 5.6 mg), and fraction 3 with t_R 21.5–24.0 min (for 1 h, 6.0 mg; for 24 h, 9.6 mg; and for 96 h, 14.5 mg) were obtained. Fraction 1 was dissolved in 0.8 mL of water and 0.1 mL of the mixture was subjected to RP-HPLC for further purification. This procedure was repeated eight times. Each fraction was lyophilized and then subjected to LC–MS and methylation analysis.

3.2.9. Preparation of oligosaccharides from maltoheptaose synthesized with transglucosidase

The reaction was performed as described above, except that [U–^{13}C]maltose was omitted.

Fraction 1 with t_R 16.0–19.7 min (for 1 h, 15.7 mg; for 24 h, 10.1 mg; and for 96 h, 2.7 mg), fraction 2 with t_R 19.7–21.5 min (for 1 h, 7.1 mg; for 24 h, 4.1 mg; and for 96 h, 3.5 mg), and fraction 3 with t_R 21.5–24.0 min (for 1 h, 5.2 mg; for 24 h, 8.8 mg; and for 96 h, 15.8 mg) were lyophilized. Fraction 1 was dissolved in water (0.8 mL) and was subjected to RP-HPLC for further purification. Each fraction was separated. Fraction D-8 was eluted with t_R 42 min. All the fractions were lyophilized and then subjected to LC–MS and methylation analysis.

3.2.10. Methylation analyses of oligosaccharides

Methylation was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide in accordance with the procedure of Ciucanu and Kerek,[18] modified as reported by Needs and Selvendran,[19] except that the reaction was performed with a microwave reaction apparatus[24] (Discover, CEM, USA) at 30 °C for 0.5 h. The methylated products were hydrolyzed with 0.5 M HCl/aqueous 80% AcOH at 70 °C for 0.5 h, followed by reduction and acetylation to obtain partially methylated alditol acetates (PMAAs). PMAAs were identified by their fragment ions in EI-MS and by their relative retention times in GC. Their molar ratios were estimated from the peak areas of total ion chromatograms from GC–MS. Relative molar ratios of [U–^{13}C]PMAA from [U–^{13}C]glucosyl residue and [U-^{12}C]PMAA from [U-^{12}C]glucosyl residue ([U–^{13}C]PMAA/[U-^{12}C]PMAA) were estimated from the peak areas of their characteristic fragment ions (*m/z*) detected using selected ion monitor (SIM) mode as follows:

1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol (non-reducing end): *m/z* 209/205.
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol (1,4-linked glucose): *m/z* 237/233.
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucitol (1,6-linked glucose): *m/z* 192/189.

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