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Enzymatic synthesis of a β-D-galactopyranosyl cyclic tetrasaccharide by β-galactosidases

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Abstract—The galactosyl transfer reaction to *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→} (CTS) was examined using lactose as a donor and β-galactosidases from *Aspergillus oryzae* and *Bacillus circulans*. The *A. oryzae* β-galactosidase produced three galactosyl derivatives of CTS. The main galactosyl derivative produced by the *A. oryzae* enzyme was identified as 6-*O*-β-D-galactopyranosyl-CTS, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-[β-D-Gal*p*-(1→6)]-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}. The *B. circulans* β-galactosidase also synthesized three galactosyl-transfer products to CTS. The structure of main transgalactosylation product was 3-*O*-β-D-galactopyranosyl-CTS, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→3)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}. These results showed that β-galactosidase transferred galactose directly to the ring glucose residue of CTS.

Keywords: Cyclic tetrasaccharide; β-Galactosidase; Transgalactosylation

1. Introduction

Côté and co-workers first reported that a cyclic tetrasaccharide, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→} (abbreviated as CTS) was produced from a dextran-like polysaccharide, alternan, by its degradation enzyme.[1,2] CTS has a unique structure consisting of four glucose residues joined by alternate α-(1→3)- and α-(1→6)-linkages. Recently, we found a new enzymatic system capable of synthesizing this saccharide from maltodextrins by the joint reaction of two glycosyltransferases, 6-α-glucosyltransferase, and 3-α-isomaltosyltransferase.[3] We succeeded in the mass production of CTS from starch in high yield using both enzymes.[4,5] CTS has tolerance to the hydrolysis activity of glycosidases such as amylase or α-glucosidase; therefore, this saccharide is expected to be used as a low-calorie sweetener. X-Ray crystal structure analysis has shown that CTS has a shallow cavity in the center of its cyclic structure,[6] suggesting the possible ability to bind a small molecule. These properties of CTS open its potential for further applications, for example, a carrier in drug delivery systems or a material for affinity chromatography.

β-Galactosidase (EC 3.2.1.23) catalyzes not only the hydrolysis reaction of a β-(1→4)-galactosyl linkage but also the transfer reaction of a galactose residue.[7] This enzyme has been used for the synthesis of galactooligosaccharides, which are the transgalactosylation products of lactose. Furthermore, it has been shown to promote the growth of intestinal bifidobacteria by oral administration.[8,9]

In this paper, to develop new applications different from the specific characters of CTS, we tried to synthesize a novel heterogeneous branched CTS. This study deals with the production of β-D-galactopyranosyl CTS by transgalactosylation with β-galactosidases from *Aspergillus oryzae* and *Bacillus circulans*.

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2. Results

2.1. Transgalactosylation to CTS by β-galactosidases

Two kinds of β-galactosidase from *A. oryzae* and *B. circulans* were tested for transgalactosylation to CTS. The reaction mixture (670 μL) containing the enzyme (0.3 U), CTS (100 mg), lactose (100 mg) in 20 mM acetate buffer (pH 4.5 for the *A. oryzae* enzyme and pH 6.0 for the *B. circulans* enzyme) was incubated at 40 °C for 24 h. The reaction products were analyzed by HPLC. The *A. oryzae* enzyme gave three transfer products, saccharides **1** (14.1 min RT: the HPLC-retention time abbreviated as RT), **2** (15.1 min RT), and **3** (19.1 min RT) (Fig. 1a). The reaction yields of the products were 3.0% (saccharide **1**), 0.4% (**2**), and 3.7% (**3**) of the total sugar (Table 1). On the other hand, the *B. circulans* enzyme produced saccharides **1**, **4** (19.7 min RT), and **5** (20.4 min RT) as the transfer products (Fig. 1b). The reaction yields of the products were 9.3% (saccharide **1**), 2.0% (**4**), and 0.6% (**5**) (Table 1). In the LC–MS analysis, the adduct ions with sodium ion $[M+Na]^+$ at *m/z* 833 (saccharide **1**), 972 (**2**), 833 (**3**), 972 (**4**), and 972 (**5**), respectively, were monitored with electrospray ionization mass spectrometry in the positive-ion mode. These results show that saccharides **1**–**5** are branched CTSs having DP 5, 6, 5, 6, and 6, respectively.

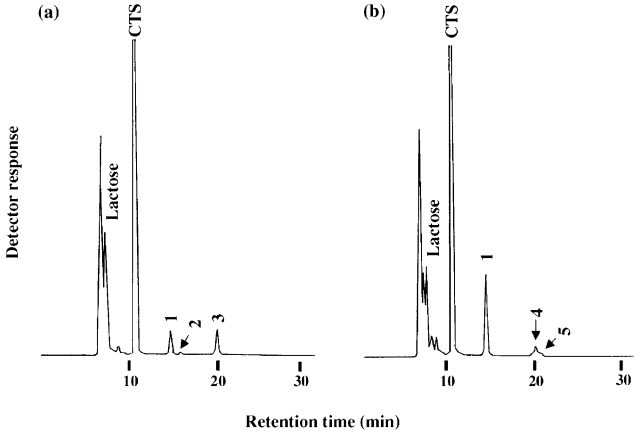


Figure 1. HPLC profile of reaction products on a mixture of lactose as the donor and CTS as the acceptor by the *A. oryzae* β-galactosidase (a), and the *B. circulans* β-galactosidase (b).

Table 1. Yields of transfer products to CTS by microbial β-galactosidase

	Yield (%)				
	1	**2**	**3**	**4**	**5**
A. oryzae	3.0	0.4	3.7	n.d.	n.d.
B. circulans	9.3	n.d.	n.d.	2.0	0.6

n.d. not detected.

2.2. Reaction yields of the transgalactosylation products

To investigate the transgalactosylation to CTS, we focused on the main products, saccharides **1** and **3**. Before preparation of the products, we examined the reaction conditions, such as the enzyme dosages, the molar ratio of CTS to lactose, and the substrate concentrations.

2.2.1. Effects of enzyme dosages. Enzyme dosages in a range from 0.3 to 300 U/g-lactose were tested. The reaction yields of saccharides **1** and **3** by the *A. oryzae* enzyme reached the maximums (3.0% and 5.8%) at the dosages of 3 and 10 U/g-lactose, respectively (Fig. 2a). Excess dosage of the enzyme decreased the yields of both products. The *B. circulans* enzyme produced saccharide **1** in a maximum yield of 9.3% when the dosage was 3 U/g-lactose, which was identical with that of the *A. oryzae* enzyme (Fig. 2b). The dosages more than 3 U/g-lactose of the *B. circulans* enzyme gave small amounts of saccharide **3**, and greater amounts of enzyme (100 and 300 U/g-lactose) produced saccharide **3** rather than saccharide **1**.

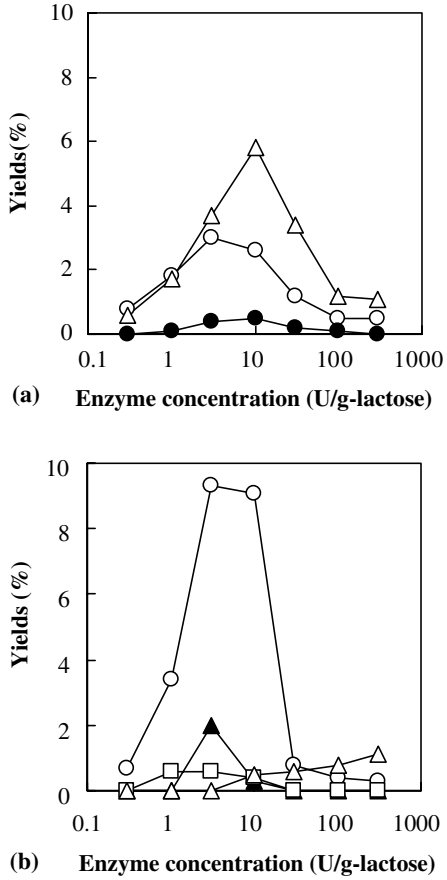


Figure 2. Effects of enzyme concentration on formation of galactosyl-CTSs by the *A. oryzae* β-galactosidase (a), and the *B. circulans* β-galactosidase (b): (○) saccharide **1**, (●) saccharide **2**, (△) saccharide **3**, (▲) saccharide **4**, (□) saccharide **5**.

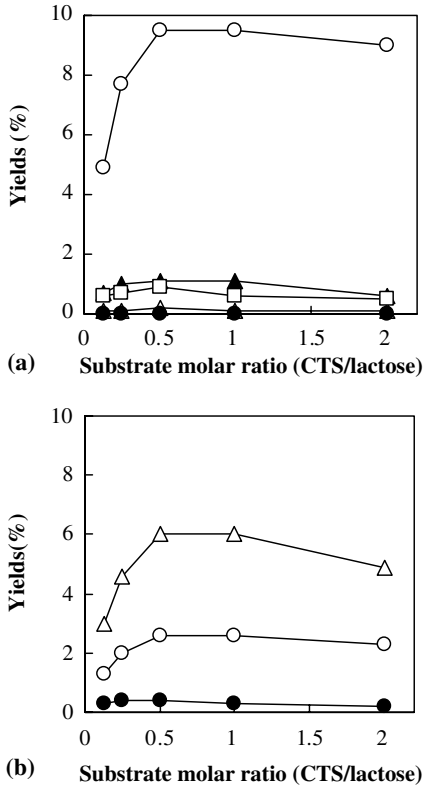


Figure 3. Effects of the ratio of CTS to lactose on formation of galactosyl-CTSs by the *A. oryzae* β-galactosidase (a), and the *B. circulans* β-galactosidase: (○) saccharide **1**, (●) saccharide **2**, (△) saccharide **3**, (▲) saccharide **4**, (□) saccharide **5**.

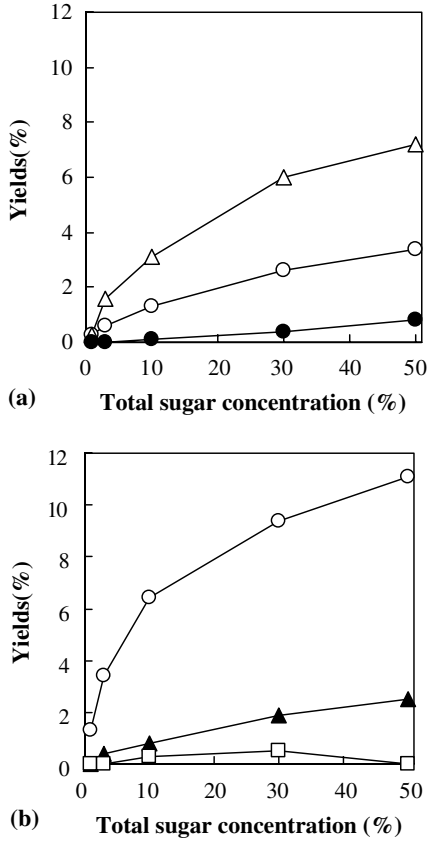


Figure 4. Effects of the total sugar concentration on formation of galactosyl-CTSs by the *A. oryzae* β-galactosidase (a), and the *B. circulans* β-galactosidase (b): (○) saccharide **1**, (●) saccharide **2**, (△) saccharide **3**, (▲) saccharide **4**, (□) saccharide **5**.

2.2.2. Effects of the molar ratio of CTS to lactose. The molar ratio of CTS to lactose in a range from 1:8 to 1:0.5 were examined. The *A. oryzae* enzyme (10 U/g-lactose) and the *B. circulans* enzyme (3 U/g-lactose) were independently incubated (total sugar concentration was fixed to 30%). As shown in Figure 3a, when the ratios of CTS to lactose were 1:2 and 1:1, the yield of saccharide **3** by the *A. oryzae* enzyme reached the maximum. Similarly to the *A. oryzae* enzyme, the maximum yield of saccharide **1** by the *B. circulans* enzyme was obtained as 1:2 and 1:1 of the ratio of CTS to lactose (Fig. 3b).

2.2.3. Effects of substrate concentrations. Substrate concentrations in a range from 1% to 50% (w/w) were studied. Each substrate containing CTS and lactose in a weight ratio of 1:1 was reacted with the *A. oryzae* enzyme (10 U/g-lactose) or the *B. circulans* enzyme (3 U/g-lactose). In cases of both the *A. oryzae* and the *B. circulans* enzymes, the yields of the transfer products increased as the substrate concentration increased (Fig. 4). The *A. oryzae* enzyme produced saccharides **1** and **3** in yields of 3.4% and 7.2% at a substrate concentration of 50%. The *B. circulans* enzyme gave saccharide **1** in a maximum yield of 11.1%. Saccharide **3** was negligibly produced from any substrate concentrations used in this experiment by the enzyme dosage of 3 U/g-lactose.

2.3. Preparation of saccharide 1

The *B. circulans* enzyme was used for preparation of saccharide **1**. A reaction mixture containing 32.2 mg of the enzyme (60 U), 20 g of lactose, 20 g of CTS, and 40 g of 20 mM sodium acetate buffer (pH 6.0) was incubated at 40 °C for 24 h and was then boiled for 10 min. To degrade the reducing saccharides, an alkaline treatment was done as follows: 4.8 g of NaOH was added into the mixture and boiled for 1 h. The treatment mixture was desalted with ion-exchange resins, 55 mL of Diaion SK1B (Mitsubishi Chem. Co., Tokyo, Japan), 50 mL of Diaion WA30 (Mitsubishi Chem. Co., Tokyo, Japan), and 80 mL of Amberlite IRA411S (Japan Organo, Tokyo, Japan), and it was then concentrated to about 50% (w/w) by evaporation. The resulting saccharide mixture contained 69.1% of CTS, 21.4% of saccharide **1**, 4.7% of saccharide **4**, 1.4% of saccharide **5** of 14.5 g of dry solid (DS). To purify saccharide **1**, a preparative ODS column chromatography on a YMC-Pack ODS-A R-355-15 120A column (5 cm i.d. × 50 cm; YMC, Kyoto, Japan)

was performed, eluting with water at a flow rate of 30 mL/min at 35 °C. After 12 cycles of chromatography injecting 1.2 g DS of samples per cycle, the purified saccharide **1**, in 100% purity, was obtained in a yield of 2.0 g.

2.4. Preparation of saccharide 3

The *A. oryzae* enzyme was used for preparation of saccharide **3**. A reaction mixture containing 87.7 mg of the enzyme (200 U), 20 g of lactose, 20 g of CTS, and 40 g of 20 mM sodium acetate buffer (pH 4.5) was incubated at 40 °C for 24 h. The reaction mixture was heated, alkali-treated, and desalted by the same procedure as that for saccharide **1**. The mixture contained 76.6% of CTS, 5.5% of saccharide **1**, 1.2% of saccharide **2**, 13.2% of saccharide **3** of 17.2 g DS. The same ODS column chromatography (14 cycles) was performed, and saccharide **3**, in 100% purity, was obtained in a yield of 1.4 g.

2.5. Characterization of saccharide 1

Methylation of saccharide **1** gave 1 mol of 2,3,4,6-tetra-*O*-methyl product derived from a nonreducing end galactose, as shown in Table 2. Compared with CTS, 1 mol of the 2,3,4-tri-*O*-methyl product was lost, and a corresponding amount of the 2,4-di-*O*-methyl product was generated. Therefore, saccharide **1** would be expected to have a structure containing one galactose residue attached to CTS by a (1 → 3)-linkage. To confirm this structure, NMR spectroscopy measurements were done. The ^{13}C NMR spectrum of saccharide **1** contained 30 signals (Table 3), indicating that it should be a pentamer of hexose. ^{1}H–^{13}C COSY showed the β-configuration of the galactose residue in saccharide **1** that was confirmed by the *C*-1 signal of this residue at 107.6 ppm {δ ^{1}H, 4.55 (d, $J_{1,2}$ 7.4 Hz)}. The large downfield shift (8.2 ppm) of the *C*-3 signal of one of the 6-*O*-glycosylated ring residues was observed in the spectra. From these results, saccharide **1** was concluded to be 3-*O*-β-D-galactopyranosyl-CTS, *cyclo*-{→6)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-[β-D-Gal*p*-(1 → 3)]-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 →} (Fig. 5a).

2.6. Characterization of saccharide 3

Methylation analysis (Table 2) indicated that saccharide **3** has a structure containing one galactose residue attached to CTS by a (1 → 6)-linkage. The ^{13}C NMR and ^{1}H–^{13}C COSY showed the β-configuration of the galactose residue that was confirmed by the *C*-1 signal of this residue at 106.0 ppm {δ ^{1}H, 4.42 (d, $J_{1,2}$ 7.5 Hz)}. The large downfield shift (7.7 ppm) of *C*-6 signal of one of the 3-*O*-glycosylated ring residue was observed (Table 3). From these results, saccharide **3** was concluded to be 6-*O*-β-D-galactopyranosyl-CTS, *cyclo*-{→6)-α-D-Glc*p*-(1 → 3)-[β-D-Gal*p*-(1 → 6)]-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 →} (Fig. 5b).

Table 2. Methylation analysis of saccharides **1** and **3**

Saccharide	2,3,4,6-Tetra-*O*-methyl (Gal)	2,3,4-Tri-*O*-methyl (Glc)	2,4,6-Tri-*O*-methyl (Glc)	2,4-Di-*O*-methyl (Glc)
1	0.9	1.0	1.9	1.2
3	1.0	2.0	1.0	1.0
CTS	—	2.0	2.0	—

Table 3. ^{13}C NMR chemical shift data for saccharides **1** and **3**[a]

Residue[b]	Carbon atom	**1**	**3**	CTS[c]
I	C-1	99.2	99.4	99.0
	C-2	74.3	74.2	74.0
	C-3	75.4	75.4	75.1
	C-4	73.3	73.2	73.0
	C-5	72.7	72.7	72.5
	C-6	70.1	70.2	69.9
II	C-1	101.0	101.2	100.8
	C-2	72.5	72.5	72.3
	C-3	77.0	77.2	76.9
	C-4	73.5	73.3	73.3
	C-5	74.2	74.2	73.9
	C-6	62.9	62.9	62.5
III	C-1	99.0	99.4	99.0
	C-2	73.7	74.2	74.0
	C-3	83.6	75.4	75.1
	C-4	73.1	73.2	73.0
	C-5	72.4	72.8	72.5
	C-6	70.1	70.4	69.9
IV	C-1	101.0	101.3	100.8
	C-2	72.6	72.6	72.3
	C-3	77.3	77.4	76.9
	C-4	73.6	73.3	73.3
	C-5	74.2	73.5	73.9
	C-6	62.9	70.6	62.5
V	C-1	107.6	106.0	
	C-2	74.1	73.6	
	C-3	75.2	75.4	
	C-4	71.5	71.3	
	C-5	78.0	77.8	
	C-6	64.2	63.7	

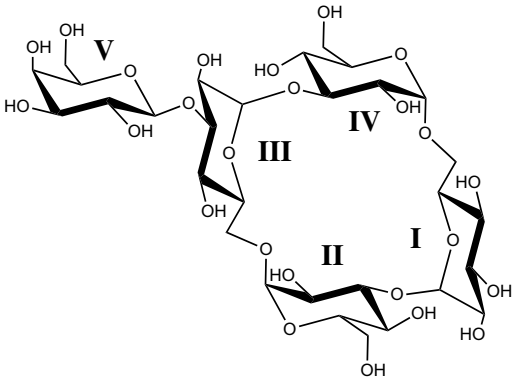
[a] NMR spectra data were recorded for solutions in D_2O at 27 °C. Chemical shifts are expressed in ppm downfield from the signal of 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (TPS), which was used as the internal standard.

[b] Roman numerals indicate the positions of the hexose residues in saccharides **1** and **3** shown in Figure 5.

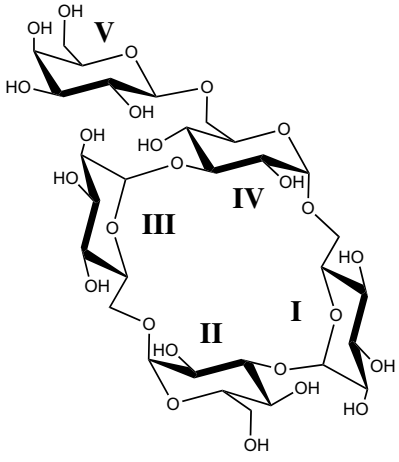
[c] Côté et al.[1]

3. Discussion

In this study, we found out that β-galactosidases from *A. oryzae* and *B. circulans* transferred galactose directly to the ring glucose residues of CTS. The *A. oryzae* enzyme produced 6- and 3-*O*-β-D-galactopyranosyl-CTS. When the enzyme dosage was more than 3 U/g-lactose, this enzyme preferentially formed 6-*O*-β-D-galactopyr-



a. Saccharide 1



b. Saccharide 3

Figure 5. Structure of saccharides **1** and **3**.

anosyl-CTS. This may be due to the fact that in the high enzyme dosage 3-*O*-β-D-galactopyranosyl-CTS is hydrolyzed easily in comparison with 6-*O*-β-D-galactopyranosyl-CTS. The molecular mass of saccharide **2**, as a minor transgalactosylated product, was found to be 972. This value identical to a doubly galactosylated CTS. The saccharide **2** would be generated with transgalactosylation to saccharide **1** or **3**.

The B. circulans enzyme catalyzed transgalactosylation mainly on *O*-3 of the α-(1→6)-glycosylated glucose residue of CTS. However, the yield of 3-*O*-β-D-galactopyranosyl-CTS noticeably decreased in over 10 U/g-lactose enzyme dosage. This result indicates that 3-*O*-β-D-galactopyranosyl-CTS is hydrolyzed in that enzyme dosage similarly to the *A. oryzae* enzyme. The molecular weights of saccharides **4** and **5** were 972. Presumably these minor products were produced by transgalactosylation to saccharide **1**.

Various β-galactosyl cyclic glucans were synthesized by using the transgalactosylation activity with β-galactosidase. Kitahata et al. attempted to transfer a galactose to cyclomalto-oligosaccharides (CDs) and their branched derivatives by various β-galactosidases.[10] Their experiments showed that the galactose-transfer products were generated from the branched derivatives such as glucosyl-CDs and maltosyl-CDs but not from the intact CDs. From the results of the structural analyses of the galactose-linked branched CDs, the position of the galactose transfer by the *B. circulans* and the *Penicillium multicolor* enzymes was identified to be 4- and 6-OH of the nonreducing glucose residue of the branched part, respectively.[11] Koizumi et al. studied the transfer of galactose to cycloisomalto-octaose (CI_8).[12] They obtained two β-D-galactopyranosyl derivatives: 2- and 3-OH of the CI_8 ring glucose residue. The *B. circulans* enzyme transferred a galactose residue onto only the *O*-2 position of the CI_8. On the other hand, the *P. multicolor* enzyme produced 3- and 2-*O*-β-D-galactopyranosyl-CI_8. When CTS was used as an acceptor, the *A. oryzae* enzyme catalyzed transgalactosylation to both the 6- and 3-OHs of CTS, but the *B. circulans* enzyme produced only 3-*O*-β-D-galactopyranosyl-CTS. The crystal structure of CTS showed that the free 3-, 4-, 6-OH and two 2-OHs, except the inner two 2-OHs of the residues, were exposed to the solvent space.[6] However, 2- and 4-*O*-β-D-galactopyranosyl-CTS were not generated in this study. It seems that the free 4-OH and the outer two 2-OHs are inaccessible to the active center of β-galactosidases because of steric hindrance.

Thus we succeeded in the enzymatic synthesis of the β-galactose conjugating CTS. Recently, we reported that the synthesis of 4-*O*-α-D-glucopyranosyl and 4,4′-di-α-D-glucopyranosyl-CTS by the cyclomaltodextrin glucanotransferase from *Bacillus stearothermophilus*[13] and 3-*O*-β-*N*-acetylglucosaminyl-CTS was accomplished by lysozyme from egg white.[14] In addition, Côté and coworkers reported that 6-*O*-α-D-galactopyranosyl-CTS was produced using α-galactosidase.[15] These branched-CTSs are expected to have various functions depend on the difference of the branched position or the kind of added saccharide.

4. Experimental

4.1. Carbohydrates

CTS (purity 99.9%) was prepared from starch with 6GT and IMT as described previously.[4] Lactose was purchased from Wako Pure Chemical Industries Co., Ltd (Osaka, Japan).

4.2. Enzymes

β-Galactosidases from *A. oryzae* (trade name; LACTASE Y-AO) and *B. circulans* (trade name; BIOLACTA) were supplied from Yakult

Pharmaceutical Industry Co., Ltd (Tokyo, Japan), and Daiwa Kasei K.K. (Osaka, Japan), respectively. Both enzymes were industrial grade.

4.3. Assay of β-galactosidase activity

A reaction mixture containing 5 mL of 1% (w/v) lactose in 20 mM acetate buffer (optimum pH of each enzyme) and 0.5 mL of the enzyme solution was incubated at 40 °C for 20 min, and the glucose produced was measured by the glucose oxidase method.[16] One unit of the enzyme activity was defined as the amount of the enzyme that liberates 1.0 μmol of glucose per min under these conditions. The β-galactosidases activities from *A. oryzae* and *B. circulans* were 2280 and 1860 U/g, respectively.

4.4. Methylation analysis

Methylation analysis was performed according to the method of Hakomori.[17] Saccharide samples (100 μg) were methylated. After extraction with $CHCl_3$, the methylated sample was hydrolyzed with 90% HOAc containing 0.5 N H_2SO_4 at 80 °C for 6 h. The methylated monosaccharides were reduced with $NaBH_4$ and then acetylated with Ac_2O at 100 °C for 4 h. The resulting partially methylated alditol acetates were analyzed by GLC (GC-14B, Shimadzu Corporation, Kyoto, Japan) on a DB-5 capillary column (J&W, Folsom, CA) at 130–250 °C (5 °C/min).

4.5. High-performance liquid chromatography (HPLC)

Samples were first treated by filtration using a filter kit, KC prep dura (0.45 μm, Katayama Chemical Co., Osaka, Japan) and by deionization using a microacilyzer G0 (Asahi Chemical Co., Tokyo, Japan). The HPLC analysis was performed using a CCPM pump, an RI-8020 refractive index monitor (Tosoh Co., Ltd, Tokyo, Japan), and a C-R7A data processor (Shimadzu Corporation, Kyoto, Japan) under the following conditions: column, ODS-AQ AQ-303 (4.6 mm i.d. × 250 mm; YMC Co., Ltd); column temperature, 40 °C; mobile phase, water; flow rate, 0.5 mL/min.

4.6. LC–MS analysis

The molecular masses of the products were determined by LC–MS. The LC–MS was carried out using the same HPLC system as above interfaced to a LCQ advantage ion-trap mass analyzer (Thermo Electron Corporation, Kanagawa, Japan) fitted with an electrospray ionization (ESI) interface. The mass spectrometer was operated in the positive-ion mode; the ESI voltage was set to 5 kV, and the capillary temperature was 350 °C.

4.7. NMR measurements

NMR spectra data were recorded for 1–5% solutions in D_2O at 27 °C with a JNM-AL300 spectrometer (^1H 300.4 MHz, ^{13}C 75.45 MHz: JEOL, Tokyo, Japan). The chemical shifts are expressed in ppm downfield from the signal of 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (TPS), which was used as an internal standard.

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