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Enzymatic synthesis, isolation, and analysis of novel α- and β-galactosyl-cycloisomalto-octaoses

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Abstract

Novel branched cycloisomalto-octaoses (CI_8s) were enzymatically synthesized by trans-galactosylation with α-galactosidase from coffee bean and β-galactosidase preparations from *Penicillium multicolor* and *Bacillus circulans*, using melibiose and lactose as donor substrates, and CI_8 which is a cyclic homogeneous oligosaccharide composed of eight glucose units bound by α-(1 → 6)-linkages, as an acceptor. α-Galactosyl-CI_8s and β-galactosyl-CI_8s obtained were isolated and purified by HPLC. Their structures were elucidated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDITOFMS) and NMR spectroscopy. © 1998 Elsevier Science Ltd.

Keywords: Galactosyl-cycloisomalto-octaose; Coffee bean α-galactosidase; *Penicillium multicolor* β-galactosidase; *Bacillus circulans* β-galactosidase; Transgalactosylation; HPLC; NMR; MALDITOFMS

1. Introduction

Cycloisomalto-oligosaccharides (CIs) are cyclic, homogeneous oligosaccharides synthesized from dextran by intramolecular transglycosylation of cycloisomalto-oligosaccharide glucanotransferase. Thus far three kinds of CI, which are respectively composed of seven, eight and nine D-glucosyl residues bound by the α-(1 → 6)-linkages, are known [1]. In our previous papers, we reported that various microbial α- and β-galactosidases produced galactosyl-transfer products of branched cyclomalto-oligosaccharides (cyclodextrins, CDs) in which the galactosyl residues were linked at the side chains of branched CDs [2–4], whereas coffee bean α-galactosidase (EC 3.2.1.22) transferred galactosyl residue(s) directly onto the CD ring [4–6]. This paper deals with syntheses of novel branched cycloisomalto-octaoses by α- and β-galactosidases and isolation and analyses of α- and β-galactosyl-cycloisomalto-octaoses (α- and β-Gal-CI_8s) produced.

2. Experimental

Materials.—CI_8 that was used as an acceptor substrate was kindly donated by Kikkoman Co. Melibiose and lactose that were used as donor substrates were purchased from Wako Pure Chemical Indus-

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tries. α-Galactosidase preparation from coffee bean (suspension in 3.2 M $(NH_4)_2SO_4$, 10 U/mg protein) was purchased from Sigma Chemical Co. β-Galactosidase preparations from *Bacillus circulans* (crude preparation, 7 U/mg), and *Penicillium multicolor* (crude preparation, 60 U/mg) were donated from Daiwa Kasei Co., Ltd., and K · I Chemical Industry Co., Ltd., respectively. Reagent-grade organic solvents used for chromatography were freshly distilled before use. Water used in solvent preparations was distilled, deionized, and redistilled.

Assay of enzyme activity.—Fifty μL of an enzyme solution was incubated with 400 μL of 5 mM *p*-nitrophenyl α-galactoside (in coffee bean α-galactosidase) or 5 mM *p*-nitrophenyl β-galactoside (in β-galactosidases) in 25 mM buffer (optimum pH of each enzyme) at 40 °C for 10 min. The reaction was stopped by adding 500 μL of 0.2 M Na_2CO_3, and the *p*-nitrophenol released from the substrate was measured spectrophotometrically at 400 nm. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per min.

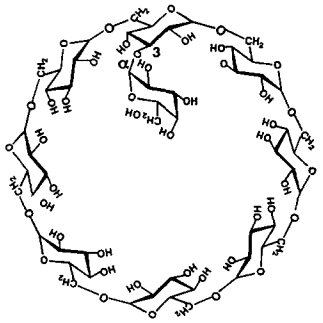
Analyses.—HPLC analyses of the reaction products were performed with a Hitachi L-6000 pump and a Hitachi 655A-30 RI monitor or a JASCO 980-PU pump and a Shodex RI-71 monitor. The columns used were a TSKgel Amide-80 (250 × 4.6 mm i.d.) (Tosoh), a YMC-pack Polyamine-II (150 × 4.6 mm i.d.) (YMC) and a DAISOPAK ODS-BP (150 × 6 mm i.d.) (Daiso). For preparative HPLC a larger size DAISOPAK ODS-BP (250 × 20 mm i.d.) (Daiso) was used. HPLC analyses at constant temperature were conducted using a column oven CO-1093C (Uniflows). A Hitachi 833A data processor was used to calculate peak areas.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDITOFMS) was performed in positive-ion mode on a Vision 2000 reflector-type TOF instrument (Thermo Bioanalysis, UK). A nitrogen laser (337 nm) with a pulse duration of 5 ns was used for ionization. 2,5-Dihydroxybenzoic acid (10 mg/mL in water) was used as the matrix. The ions generated were accelerated to a potential of 5 kV in the ion source and postaccelerated to a potential of 7 kV for detection with a secondary-ion multiplier. The MALDITOF spectra represent the accumulation of 6–10 single laser shots. They were calibrated externally by a standard sample (angiotensin I, molecular weight of 1296) that was placed on the same target. The matrix solution (0.5 μL) and the sample solution (1 mg/mL in water, 0.5 μL) were mixed and air-dried on the target.

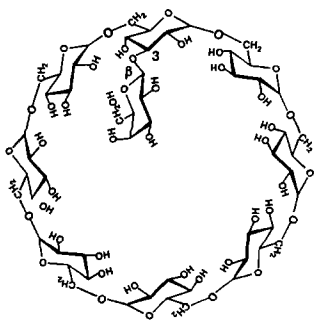
NMR spectral data were recorded for 6–10% solutions in D_2O at 50 °C with a JEOL GSX-500 spectrometer. Chemical shifts were expressed in ppm downfield from the signal of Me_4Si referenced to external 1,4-dioxane (67.40 ppm). The other conditions for ^{13}C NMR, ^1H–^1H COSY and ^1H–^{13}C COSY measurements were the same as reported in the previous paper [6].

3. Results and discussion

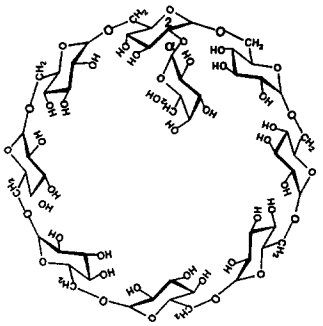
Transgalactosylation of CI_8 by α- and β-galactosidases.—Coffee bean α-galactosidase (0.5 U) was incubated with melibiose (0.6 M) as a donor substrate and CI_8 (0.1 M) as an acceptor in 100 μL of 50 mM phosphate buffer (pH 6.5) at 40 °C. After 5, 22, 34, 46 and 50 h, 3 μL samples of the reaction mixture were removed to be analyzed by HPLC. Two peaks of transgalactosylated derivatives of CI_8 [α-Gal-CI_8 (I) and (II)] were detected (Fig. 1[A]). The yields of α-Gal-CI_8 (I) and (II) were almost the same and gradually increased with increasing reaction time (Fig. 2[A]).



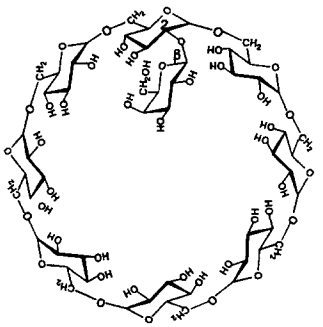
α-Gal-CI_8 (I)



β-Gal-CI_8 (I)



α-Gal-CI8 (II)



β-Gal-CI8 (II)

β-Galactosidase preparations from *P. multicolor* and *B. circulans* (20 U) were separately incubated with lactose (0.6 M) as a donor substrate and CI_8 (0.1 M) as an acceptor in 100 μL of 50 mM acetate buffer (pH 4.5 for *P. multicolor* and pH 6.0 for *B. circulans*) at 40 °C. At 0.5, 1.0, 1.5, 2.0 and 3.5 h, 3 μL of the reaction mixtures were removed to be analyzed by HPLC. In the case of *P. multicolor* β-galactosidase, two peaks of transgalactosylated derivatives of CI_8 [β-Gal-CI_8 (I) and (II)] were detected (Fig. 1[B]). The yield of β-Gal-CI_8 (I) reached a maximum value (10%) at 1.5 h and then gradually decreased. On the other hand, the yield of β-Gal-CI_8 (II) increased as the reaction proceeded (maximum yield was 5.3% at 3.5 h (Fig. 2[B]). *B. circulans* β-galactosidase produced only one transgalactosylated product of CI_8 in high yield (18%) (Fig. 1[C] and Fig. 2[C]). This product was identified by HPLC on normal-phase columns (TSKgel Amide-80 and YMC-pack Polyamine-II) and a reversed-phase column (DAISOPAK ODS-BP) as β-Gal-CI_8 (II).

Effect of substrate concentration on the synthesis of β - Gal - CI_8 (I) and (II) by P. multicolor *β - galactosidase.*—In the synthesis of transfer product using hydrolase, the yield is usually affected by the substrate concentration. Therefore the effect of substrate concentration on the synthesis of transfer products was investigated using *P. multicolor* β-galactosidase. *P. multicolor* β-galactosidase (950 U/g of lactose) was incubated with various concentrations of lactose in the presence of 0.1 M CI_8 at 40 °C. The amounts of β-Gal-CI_8 (I) and (II) in the reaction mixtures were measured by HPLC (Table 1). The yields of β-Gal-CI_8 (I) and (II) increased with increase of lactose concentration, due to efficiency of transgalactosylation being enhanced. The maximum yields of β-Gal-CI_8 (I) (9.77%) and (II) (6.74%) were obtained at the lactose concentration of 0.8 M, practically the usable maximum concentration, at 50 min and 180 min, respectively. At each lactose con-

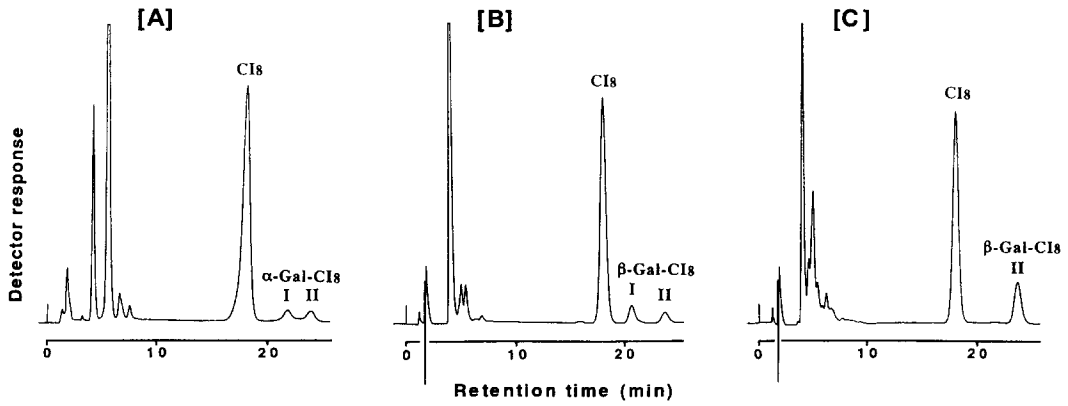


Fig. 1. Chromatograms of transgalactosylation products of cycloisomalto-octaose (CI_8) by coffee bean α-galactosidase [A], and β-galactosidases from *Penicillium multicolor* [B] and from *Bacillus circulans* [C]. Chromatographic conditions: column, YMC-Pack Polyamine II (150 × 4.6 mm i.d.); eluent, 6:4 CH_3CN–H_2O; flow rate, 1 mL/min; temperature, 30 °C.

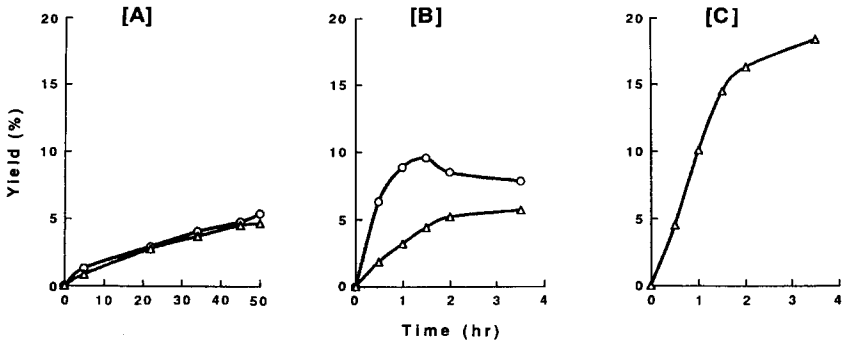


Fig. 2. Time courses of formation of transgalactosylated products by coffee bean α-galactosidase [A], and β-galactosidases from *Penicillium multicolor* [B] and from *Bacillus circulans* [C]. Symbols: ○–○ (I); △–△ (II).

Table 1
Effect of lactose concentration on the production of β-Gal-CI_8 by *Penicillium multicolor* β-galactosidase

	Yield (%)[a]					
	0.05 M		0.20 M		0.80 M	
	(I)[b]	(II)[c]	(I)[b]	(II)[c]	(I)[b]	(II)[c]
10 min	1.20	0.35	3.58	0.78	3.61	0.82
20 min	1.86	0.36	5.02	1.11	6.65	1.89
50 min	2.18	0.69	5.66	1.61	9.77	4.17
180 min	1.32	0.67	2.93	2.39	8.07	6.74
300 min	0.58	0.48	0.99	1.65	3.59	4.62

[a][Transfer product (moles)/CI_8s (moles)] × 100.
[b]β-Gal-CI_8 (I).
[c]β-Gal-CI_8 (II).

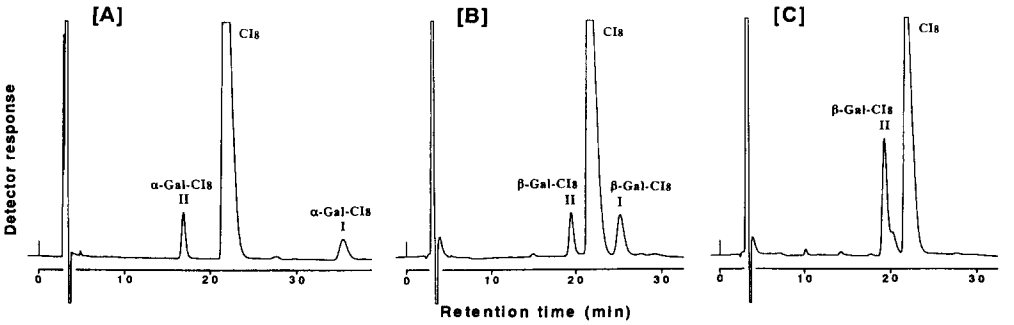


Fig. 3. Elution profiles of transgalactosylation products of cycloisomalto-octaose (CI_8) by coffee bean α-galactosidase [A], and β-galactosidases from *Penicillium multicolor* [B] and from *Bacillus circulans* [C] on an ODS column. Chromatographic conditions: column, DAISOPAK ODS-BP (150 × 6 mm i.d.); eluent, 7:93 CH_3OH–H_2O; flow rate, 1 mL/min; temperature, 30 °C.

centration, the yields of β-Gal-CI_8 (I) and (II) decreased after 50 min and 180 min, respectively, because of the hydrolysis by β-galactosidase.

Preparation of α- and β-Gal-CI_8s by α- and β-galactosidases.—Coffee bean α-galactosidase (2.4 mg, 1 mL) was added to a mixture of melibiose (3.16 g) and CI_8 (2 g) in a volume of 14.4 mL of 50 mM phosphate buffer (pH 6.5) and incubated at 40 °C for 48 h. In the preparation of β-galactosylated CI_8, β-galactosidase preparations (50 mg, 1 mL) from *P. multicolor* and *B. circulans* were separately added to a mixture of lactose (3.16 g) and CI_8 (2 g) in a volume of 14.4 mL of 50 mM acetate buffer (pH 4.5 for *P. multicolor* and pH 6.0 for *B. circulans*) and incubated at 40 °C for 3 h. The reaction mixture was heated at 100 °C for 10 min to inactivate the enzyme and centrifuged to remove the insoluble materials.

Isolation of α- and β-Gal-CI_8 [I] and [II].—At first the separation of Gal-CI_8s from a mixture of transgalactosylation products was attempted by HPLC on a TSKgel Amido-80 column (300 × 21.5 mm i.d.) with 55:45 acetonitrile–water at flow rate of 4 mL/min. The elution profiles of transgalactosylation products under this conditions were similar to those in Fig. 1. However, the loading capacity of this column was low (10 mg), and, moreover, this method was very time-consuming (about 110 min for one analysis). Therefore, the separation of Gal-CI_8s on a reversed-phase column was investigated. Fig. 3[A] shows a chromatogram of transgalactosylation products by coffee bean α-galactosidase on an ODS column for analysis with 7:93 methanol–water. α-Gal-CI_8 (II) eluted before the parent CI_8, and α-Gal-CI_8 (I) eluted at last. β-Gal-CI_8s showed similar behaviour under the same chromatographic conditions (Fig. 3[B]). Although an unknown peak is observed behind of β-Gal-CI_8 (II) as a shoulder in Fig. 3[C], and this eluted together with β-Gal-CI_8 (II) as a single peak on a YMC-pack Polyamine-II column (Fig. 1[C]), the chromatographic behaviour of this unknown peak on ODS columns was different from that of CI_8s, i.e., on a larger size column with a higher concentration of methanol in the eluent the unknown eluted before β-Gal-CI_8 (II). Therefore, this unknown was not regarded as an analog of the CI_8 derivatives. By the use of a larger size of DAISOPAK ODS column (250 × 20 mm i.d.) with 1:9 methanol–water at flow rate of 3 mL/min, α-Gal-CI_8 (I) and (II), and β-Gal-CI_8 (I) and (II) were efficiently isolated from each mixture of transgalactosylation products. In this semipreparative chromatography the loading capacity of this ODS column was 100 mg, and, moreover, the time required for one analysis was shorter than that on the above-men-

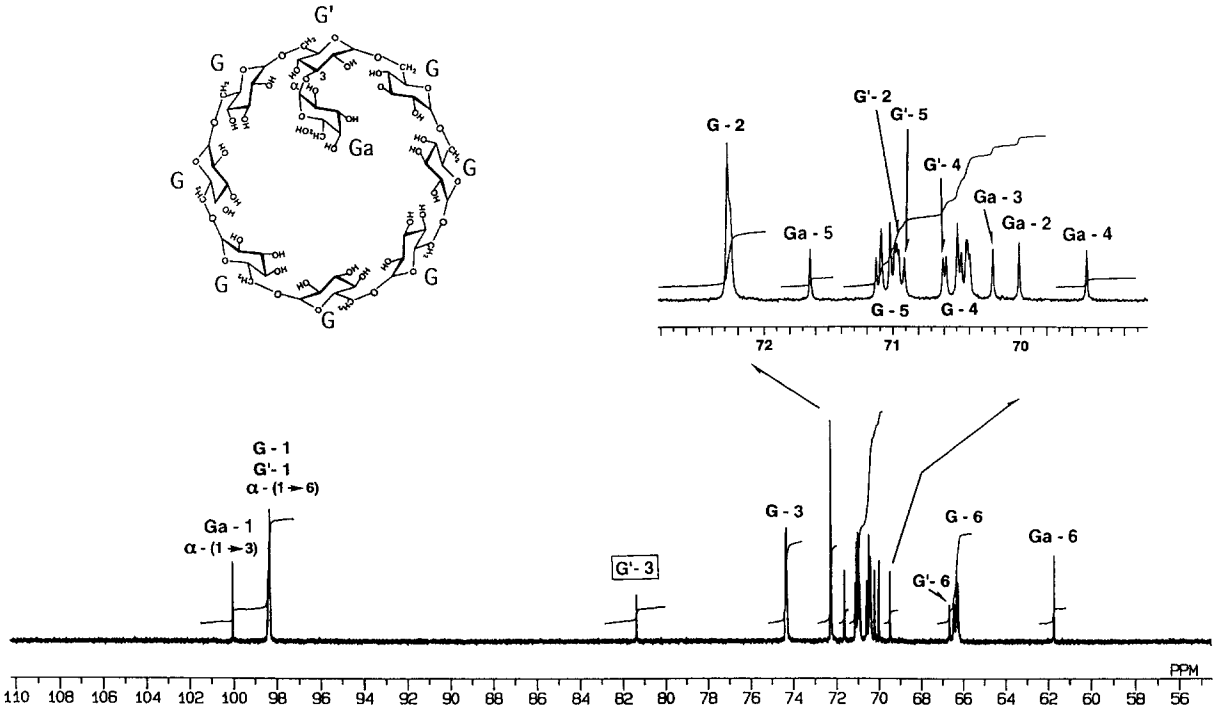


Fig. 4. ^{13}C NMR spectrum of α-Gal-CI_8 (I) in D_2O at 50 °C. G-1, -2, -3, -4, -5 and -6 are signals of C-1, -2, -3, -4, -5 and -6 atoms of the ring D-glucopyranose units. G′ is the ring D-glucopyranose unit on which galactopyranosyl residue is linked. Ga-1, -2, -3, -4, -5 and -6 are signals of C-1, -2, -3, -4, -5 and -6 atoms of the side-chain D-galactopyranose unit.

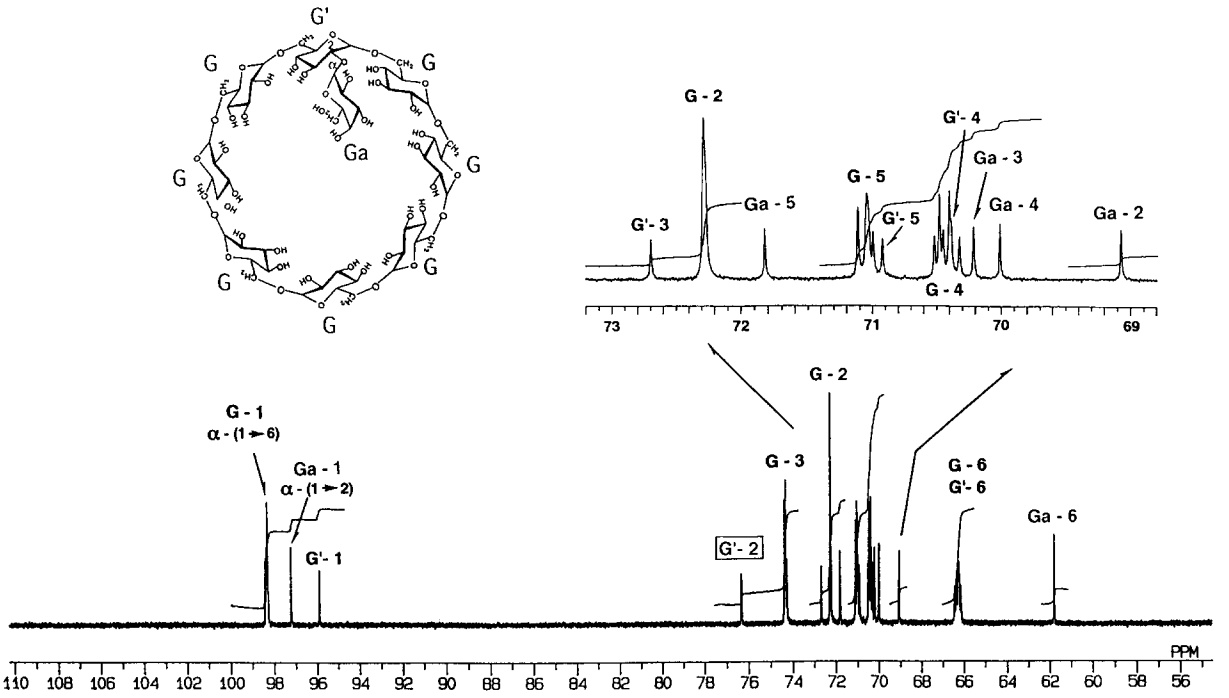


Fig. 5. ^{13}C NMR spectrum of α-Gal-CI_8 (II) in D_2O at 50 °C. Symbols are the same as in Fig. 4.

tioned TSK gel Amido-80 column in spite of the slower flow rate (within 50 min). Thus, for separation of nonreducing cyclic oligosaccharides, the ODS column is superior to the so-called amino column that is commonly used for separation of carbohydrates.

Analyses.—In each TOFMS spectrum of α- and β-Gal-CI_8 (I) and (II) the molecular-ion peak in the positive mode $[M + Na]^+$ was observed at *m/z* 1481.5. Consequently, their degree of polymerizations (dp's) were all 9; that is, they had one galacto-

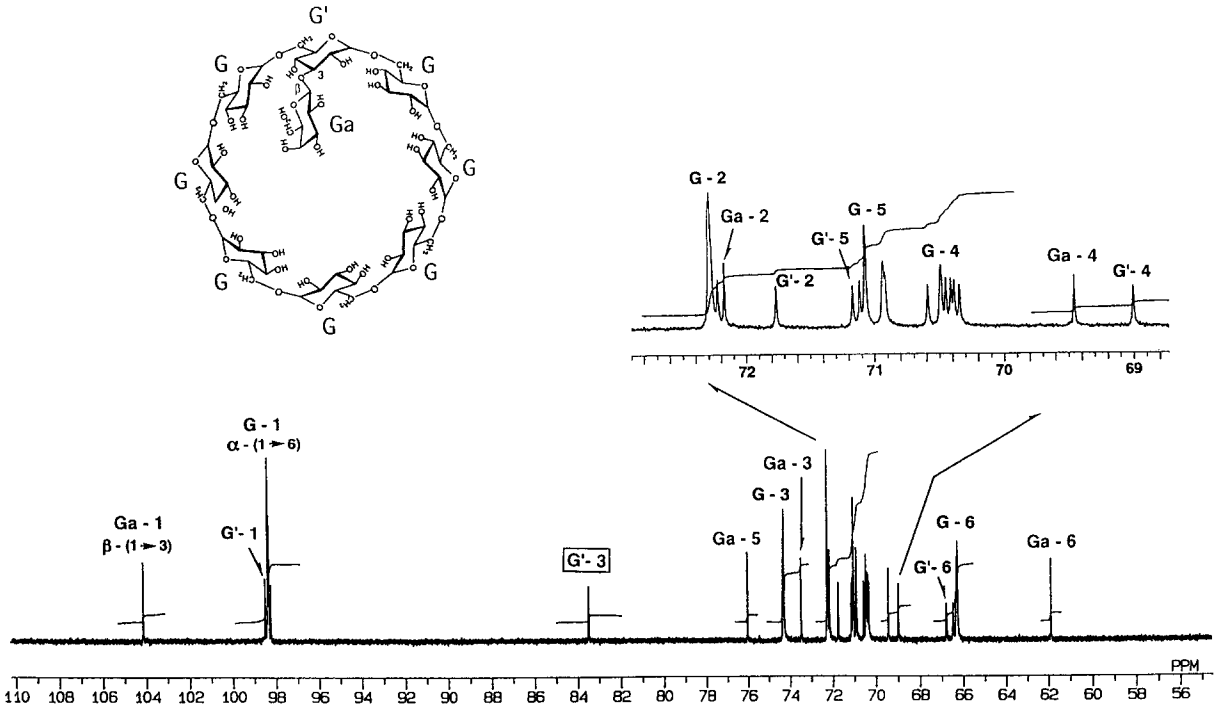


Fig. 6. ^{13}C NMR spectrum of β-Gal-CI_8 (I) in D_2O at 50 °C. Symbols are the same as in Fig. 4.

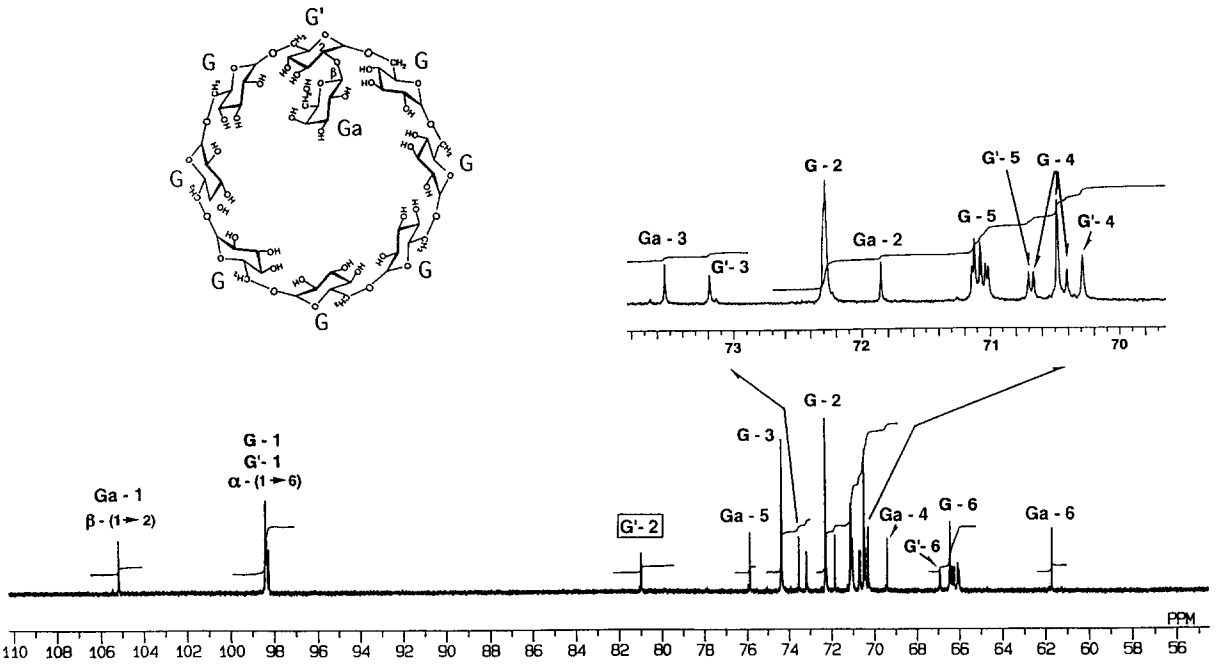


Fig. 7. ^{13}C NMR spectrum of β-Gal-CI_8 (II) in D_2O at 50 °C. Symbols are the same as in Fig. 4.

syl side chain linked directly to CI ring, and (I) and (II) were positional isomers. In addition, a trace peak (named α-Gal-CI_8 (III)) observed at $t_R = \sim 28$ min in the chromatogram of Fig. 3[A] was also collected in the semipreparative chromatography. It was confirmed by TOFMS that this α-Gal-CI_8 (III) had the same molecular weight (dp 9) as α-Gal-CI_8 (I) and (II); therefore, it was another positional isomer of α-Gal-CI_8 (I) and (II). Two minor peaks behind β-Gal-CI_8 (I) in Fig. 3[B] corresponded to di-galactosyl substituted CI_8 (dp 10) by TOFMS.

It was confirmed by NMR spectroscopy on which position of the CI ring glucose the galactosyl residue was substituted. The ^{13}C resonances of all carbons in the spectra of α- and β-Gal-CI_8 (I) and (II) were assigned using COSY and ^1H–^{13}C COSY methods. Fig. 4 shows the ^{13}C NMR spectrum of α-Gal-CI_8 (I). The shift of the C-3 signal (G′-3) of one CI ring glucose downward to δ 81.4 from other C-3 signals (G-3) of CI ring glucoses (δ 74.3 ~ 74.4) indicates a galactosyl residue substituted on the oxygen atom attached to the C-3. Consequently, α-Gal-CI_8 (I) was thought to be 3-*O*-α-D-galactopyranosyl-CI_8.

In the ^{13}C NMR spectrum of α-Gal-CI_8 (II), one signal for the C-2 of CI ring glucose (G′-2, δ 76.3) shifted downfield from the other C-2s (G-2, δ ~ 72.3) (Fig. 5). Therefore, α-Gal-CI_8 (II) was 2-*O*-α-D-galactopyranosyl-CI_8. An additional characteristic of the ^{13}C NMR spectrum of α-Gal-CI_8 (II) was the upfield shift of one C-1 signal (G′-1) of the CI ring glucose on which the galactosyl residue was attached at C-2 (β effect).

Similarly in the ^{13}C NMR spectra of β-Gal-CI_8 (I) and (II) each one C-3 signal (G′-3) and C-2 signal (G′-2) of CI ring glucose shifted downfield by ~ 9 ppm, respectively (Figs. 6 and 7), indicating that β-Gal-CI_8 (I) was 3-*O*-β-D-galactopyranosyl-CI_8 and β-Gal-CI_8 (II) was 2-*O*-β-D-galactopyranosyl-CI_8. In the case of the latter the upfield shift of one C-1 signal (G′-1) by β effect was not observed unlike 2-*O*-α-D-galactopyranosyl-CI_8.

4. Conclusions

α-Galactosidase from coffee bean transferred one galactosyl residue to C-2 or C-3 of one CI ring glucose with almost the same probability. Furthermore, as the production of another positional isomer was suggested by HPLC and TOFMS, transgalactosylation to C-4 of CI ring glucose had occurred, but with very low probability. In the transgalactosylation of cyclomaltooligosaccharides (cyclodextrins, CDs), which have α-(1 → 4)-linkages and form hydrogen bonds between hydroxy groups on C-2 of a ring glucose and C-3 of the adjacent glucose residue, this α-galactosidase mainly produced 6-*O*-α-D-galactopyranosyl-CD, while 2-*O*-α-D-galactopyranosyl-CD was produced as a minor product [6]. It is understandable that transgalactosylation of CI by coffee bean

α-galactosidase occurred mainly on C-2 and C-3 of CI ring glucose, since CI has α-(1 → 6)-linkages and probably lacks hydrogen bonds between hydroxyl groups on C-2 and C-3 of adjacent glucoses.

In contrast, microbial β-galactosidases did not transfer a galactosyl residue directly onto the CD ring [2,3], whereas these enzymes catalyzed direct transgalactosylation on CI ring glucose. β-Galactosidase from *P. multicolor*, which transferred a galactosyl residue on C-6 of side chain glucose of glucosyl- or maltosyl-CDs [3], preferentially produced C-3 galactosylated CI_8, and produced also C-2 galactosylated CI_8.

β-Galactosidase from *B. circulans*, which catalyzed transgalactosylation mainly on C-4 and also on C-6 of side chain glucose of glucosyl- or maltosyl-CDs [3], synthesized only C-2 galactosylated CI_8. Compared with the cyclic structure of CD ring made up of a glucan having α-(1 → 4)-linkages, a CI ring made up of a glucan having α-(1 → 6)-linkages may take a linear-like structure and so microbial β-galactosidases could transfer a galactosyl residue directly onto the CI ring glucose. However, microbial β-galactosidases transferred the galactosyl residue to the hydroxyl group on C-2 or C-3 of CI ring glucose, but not to C-4 of CI ring glucose, probably because of steric hindrance.

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