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Isolation and characterization of novel heterogeneous branched cyclomalto-oligosaccharides (cyclodextrins) produced by transgalactosylation with α-galactosidase from coffee bean

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Abstract

Transgalactosylated derivatives of cyclomalto-hexaose (αCD), -heptaose (βCD), and -octaose (γCD) were synthesized by α-galactosidase from coffee bean using melibiose as a donor and αCD, βCD or γCD as an acceptor. Mono- and di-*O*-α-D-galactosylated CDs were isolated and purified by HPLC. Their structures were elucidated by fast-atom bombardment mass spectrometry (FABMS) and ^{13}C NMR spectroscopy. For structural determination of positional isomers of $6^1,6^n$-di-*O*-α-D-galactosyl-CDs, digestion products with cyclodextrin glucanotransferase were analyzed by HPLC and FABMS.

Keywords: Galactosyl cyclodextrin; α-Galactosidase, coffee bean; Transgalactosylation; HPLC; ^{13}C NMR; FABMS

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1. Introduction

To develop new applications different from those of conventional cyclomalto-oligosaccharides (cyclodextrins, CDs) and homogeneous branched CDs, we have tried to synthesize heterogeneous branched CDs, which are expected to be useful as possible drug carriers in drug delivery systems. In our previous papers [1–3], we reported that various microbial α- and β-galactosidases produced galactosyl-transfer products of branched CDs in which the galactosyl residues were linked at the side chains of branched CDs. Recently, we found that coffee bean α-galactosidase (EC 3.2.1.22) transferred a galactosyl residue directly on to the CD ring, and we have since synthesized novel branched CDs [3,4]. This study deals with isolation and characterization of these novel mono-*O*-α-D-galactosyl-CDs together with those of di-*O*-α-D-galactosyl-CDs newly found. Di-*O*-α-D-galactosyl-CDs may be more interesting from the standpoint of cluster ligands for animal lectin studies [5].

2. Experimental

Materials.—αCD, βCD, and γCD were supplied by Ensuiko Sugar Refining Co., Ltd. Melibiose was purchased from Wako Pure Chemical Industries. Coffee bean α-galactosidase preparation (12 units/mg protein) was purchased from Sigma Chemical Co. and used after dialysis against 50 mM acetate buffer (pH 6.5). In order to use for production of galactosyl-βCD on an industrial scale, a crude α-galactosidase was prepared from green coffee beans as follows: 38.5 kg of green coffee beans (''Mocha'' brand) were pulverized in a coffee mill. The powder was suspended in 200 L of 0.9% sodium chloride solution and stirred mechanically overnight at 5°C. Ammonium sulfate (99 kg) was added to the solution (160 L), and the mixture was stirred mechanically. After one night precipitates were collected by continuous centrifugation (4°C, 9000 rpm) and were dissolved in 20 L of water. α-Galactosidase activity of this solution was 86,240 units (4.3 units/mL). Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) (1200 units/mL) from *Bacillus stearothermophilus* was prepared and purified as previously described [6]. Reagent-grade organic solvents used for chromatography were freshly distilled before use. Water used in solvent preparations was distilled, deionized, and redistilled.

Preparation and isolation of α-D-galactosylated CDs.—According to the methods described in the previous paper [3], coffee bean α-galactosidase was incubated with a mixture of melibiose as a donor and αCD, βCD or γCD as an acceptor in 50 mM acetate buffer (pH 6.5) at 40°C for 48 h. The reaction mixtures were heated at 100°C for 15 min to inactivate the enzyme and were then centrifuged to remove the insoluble materials. After removing the mono- and acyclic oligo-saccharides from the reaction mixture by preparative HPLC on an ODS column (1000 × 26.4 mm i.d.) with water, CDs were eluted from the column with 30% ethanol. Mono- and di-*O*-α-D-galactosyl-CDs were fractionated from the mixture of CDs by HPLC on a TSKgel Amide-80 column (300 × 21.5 mm i.d.) with 58% acetonitrile. Components in each mixture of mono- and di-galactosylated CDs were isolated by HPLC on a YMC-Pack SH-343-5 ODS column

(5 μm, 250 × 20 mm i.d.) with 5:95–8:92 methanol–water and purified on a YMC-Pack A-323-3 ODS column (3 μm, 250 × 10 mm i.d.) with 4:96–6:94 methanol–water or on a graphitized carbon column, Carbonex (100 × 10 mm i.d.) with 9:91 acetonitrile–water.

Analyses.—HPLC analyses were performed with a JASCO 980-PU pump and a Shodex RI-71 monitor. The columns used were a YMC-Pack A-312-3 (3 μm, 150 × 6 mm i.d.) (YMC), a Carbonex (100 × 4.6 mm i.d.) (TONEN), and a YMC-pack Polyamine-II (250 × 10 mm i.d.) (YMC). HPLC analyses at constant temperature were conducted using a column oven CO-1093C (Uniflows).

FABMS was performed with a JEOL JMS-DX 303 mass spectrometer using Xe atoms having a kinetic energy equivalent to 6 kV at an accelerating voltage of 3 kV. The mass marker was calibrated with perfluoroalkylphosphazine (Ultra Mark), and glycerol was used as the matrix solution.

NMR spectra 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 in the previous paper [2].

Structural analyses of positional isomers by enzymic degradation.—A sample (0.2 mg) of 6^1,6$^{''}$-di-*O*-α-D-galactosyl-αCDs (**2a**, **2b**, and **3**) and -βCDs (**IIa**, **IIb**, and **IIc**) in 70 μL of 20 mM acetate buffer (pH 6.0) was incubated with CGTase (36 units) at 40°C for 24 h, and the enzyme was inactivated by keeping it in a boiling water bath for 10 min. Each hydrolysate with enzymic degradation was analyzed by HPLC on a YMC-Pack Polyamine-II column and then by FABMS.

3. Results and discussion

Preparation.—From each CD, two kinds of mono-galactosylated products (**A**: major and **B**: minor, Fig. 1) and small amounts of digalactosylated products were synthesized. In Table 1 their yields (%) are listed. When the transgalactosylation with coffee bean α-galactosidase was carried out with higher concentration (e.g., 1 M) of the donor substrate for longer times (72 h), the proportions of digalactosyl-αCDs and -βCDs increased up to about 10% of monogalactosyl-αCDs and -βCDs, respectively. The ratios of components in each mixture of digalactosyl-αCDs and digalactosyl-βCDs measured from their chromatograms (Figs. 2 and 3-[1], respectively) are listed in Tables 2 and 3. In addition, those of digalactosyl-βCDs prepared using a crude α-galactosidase extracted from green coffee beans, calculated from the peak areas in Fig. 3-[2], are also shown in Table 3.

Isolation.—Fig. 1 shows chromatograms of monogalactosylated αCD (Gal-αCD), βCD (Gal-βCD), and γCD (Gal-γCD). The minor product **B** is eluted faster than the major one **A** in the chromatogram of Gal-αCD, whereas in the cases of Gal-βCD and Gal-γCD, the elution orders are reversed. Those two components could be separated by semipreparative HPLC on a YMC-Pack ODS A SH-343 column with methanol–water.

Analysis of a mixture of digalactosyl-αCDs by HPLC on an ODS column showed the presence of several kinds of components (Fig. 2), and, moreover, peak 2 was

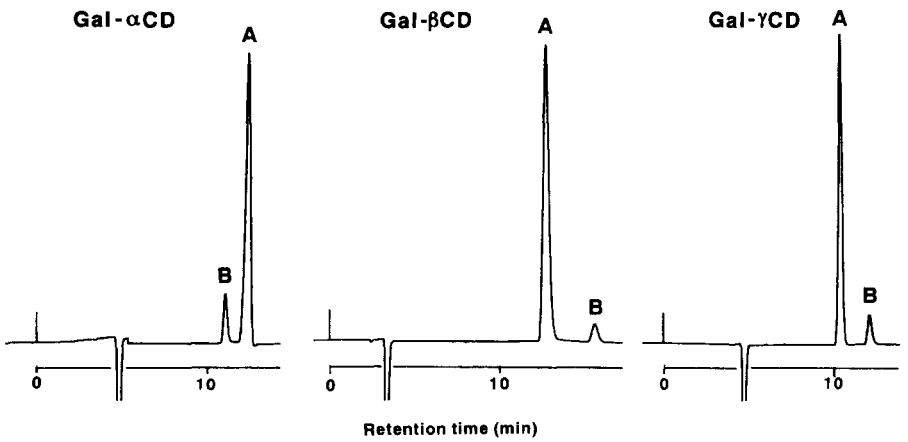

Fig. 1. Chromatograms of monogalactosyl-CDs. **A** = 6-*O*-α-D-galactopyranosyl-CD, **B** = 2-*O*-α-D-galactopyranosyl-CD. Chromatographic conditions: column, YMC-Pack A-312-3 (150×6 mm i.d.); eluent, CH_3OH–H_2O (6:94 for Gal-αCD and Gal-γCD, 7:93 for Gal-βCD); flow rate, 0.7 mL/min for Gal-αCD and Gal-γCD, 1.0 mL/min for Gal-βCD; temperature, 30°C.

separated into two peaks (2a and 2b) on an graphitized carbon column (the insert in Fig. 2). All components were isolated by semipreparative HPLC using a larger ODS column with methanol–water and a carbon column with acetonitrile–water.

Fig. 3-[1] shows separation of digalactosyl-βCDs prepared using the commercial enzyme preparation. Peaks II and III were fractionated, and rechromatography of each fraction with an eluent containing a lower concentration of methanol resulted in separation into three peaks (IIa, IIb, and IIc) and five peaks (IIIa, IIIb, IIIc, IIId, and IIIe), respectively.

Another mixture of digalactosyl-βCDs produced by a crude α-galactosidase, which was prepared from green coffee beans in the Carbohydrate Research Laboratory of Ensuiko Sugar Refining Co. Ltd., showed a very different elution profile (Fig. 3-[2]) from that of digalactosyl-βCDs prepared using the commercial enzyme (Fig. 3-[1]).

Table 1
Yields (%) of transfer products (monogalactosyl-CDs, **A** and **B**, and a mixture of digalactosyl-CDs) to CD from melibiose with coffee bean α-galactosidase [a]

Reaction time (h)	αCD			βCD			γCD		
	mono(**A**)	mono(**B**)	di(mix)	mono(**A**)	mono(**B**)	di(mix)	mono(**A**)	mono(**B**)	di(mix)
1	8.29	2.05	0.34	13.23	0.80	0.01	10.48	0.51	0.12
6	16.15	2.16	0.66	23.40	1.34	0.43	18.88	1.31	1.21
20	22.65	1.74	0.95	26.48	1.34	1.02	22.35	1.69	2.17
48	23.45	1.47	1.42	23.71	1.12	1.66	20.43	1.77	2.51
72	23.07	1.33	1.41	20.74	0.95	1.70	18.23	1.50	2.26

[a] Reaction conditions: CD 0.1 M, melibiose 0.2 M, α-galactosidase 36U/melibiose 1 g, pH 6.5, 40°C. Yield (%) = [Transfer product (mol)/Total CDs (mol)] × 100.

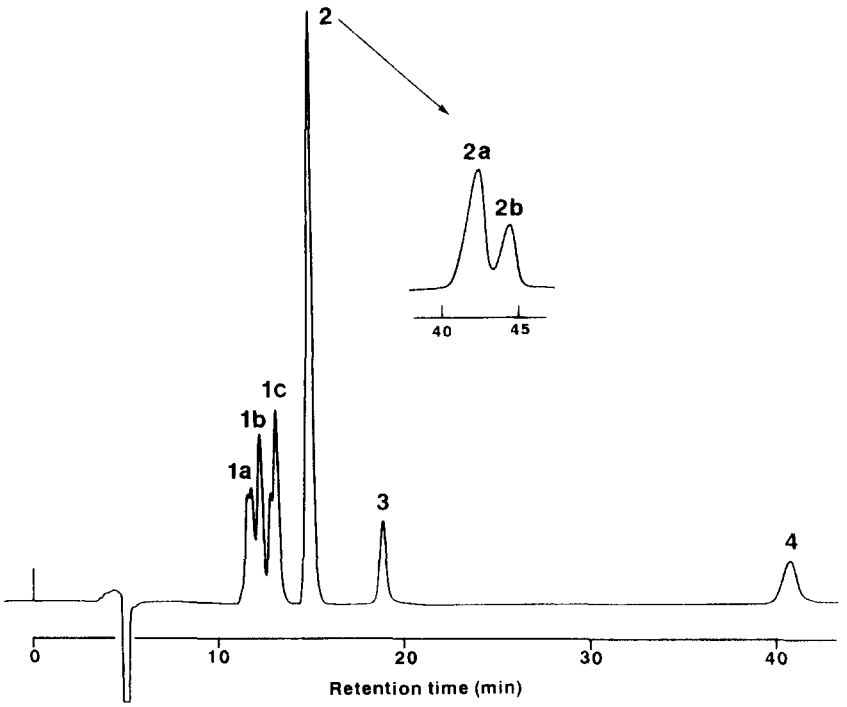

Fig. 2. Elution profile of a mixture of digalactosyl-αCDs. Chromatographic conditions: eluent, 4:96 CH_3OH–H_2O; flow rate, 0.7 mL/min; other conditions as in Fig. 1. Conditions for separation of **2a** and **2b**: column, Carbonex (100×4.6 mm i.d.); eluent, 1:9 CH_3CN–H_2O; flow rate, 1.5 mL/min; temperature, 30°C.

Digalactosyl-βCDs were roughly separated by HPLC on a YMC-Pack A SH-343-5 column and purified by rechromatography on a YMC-Pack A-323-3 column.

Characterization.—(1) Monogalactosyl-CDs. In the preceding paper [3] it was established by α-galactosidase digestion and by FABMS and ^{13}C NMR spectroscopies that the major product **A** of monogalactosylation to each CD was 6-*O*-α-D-galactopyranosyl-CD. The minor product **B** was predicted to be a positional isomer of **A**, since **B** gave the same results as **A** by α-galactosidase digestion and FABMS spectroscopy; that is, **B** was hydrolyzed with coffee bean α-galactosidase to galactose and the parent CD in the molar ratio of 1:1, and the FABMS spectra of Gal-αCD (**B**), Gal-βCD (**B**), and Gal-γCD (**B**) in the negative mode exhibited the molecular ion $[M - H]^-$ peak and each one fragment ion $[M - Gal - H]^-$ peak at m/z 1133 and 971, 1295 and 1133, and 1457 and 1295, respectively. In order to determine the position substituted by the galactosyl residue, the ^{13}C NMR resonance of all carbons in the spectrum of Gal-αCD (**B**) were assigned using ^1H–^1H COSY and ^1H–^{13}C COSY methods. Fig. 4 shows the ^1H–^{13}C COSY spectrum of Gal-αCD (**B**) in D_2O at 50°C. The spectra of Gal-βCD (**B**) and Gal-γCD (**B**) were similar to that of Gal-αCD (**B**), and hence assignments of signals could be made by analogy. It is known that a substituent on the oxygen atom attached to any carbon of the sugar affects the chemical

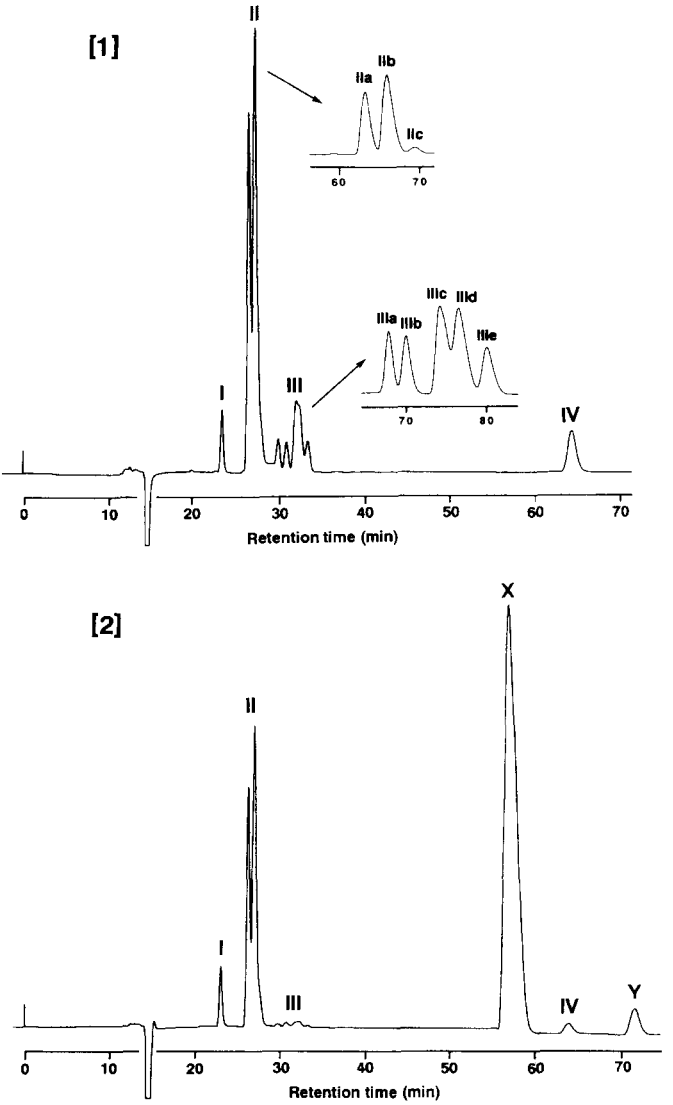

Fig. 3. Elution profiles of a mixture of digalactosyl-βCDs produced with the commercial coffee bean α-galactosidase [1] and a mixture of digalactosyl-βCDs produced with a crude coffee bean α-galactosidase [2]. Chromatographic conditions: column, YMC-Pack A-323-3 (250 × 10 mm i.d.); eluent, 8:92 CH_3OH–H_2O; flow rate, 1 mL/min; temperature, 30°C. Special conditions for separation of each positional isomers of **II** and **III**: eluent, 5:95 CH_3OH–H_2O.

Table 2
Ratios of isomers in a mixture of digalactosyl-αCDs

1a	1b	1c	2a	2b	3	4
9.0	9.1	14.2	34.7	16.3	9.2	7.5

Table 3
Ratios of isomers in a mixture of digalactosyl-βCDs

	I	IIa	IIb	IIc	III	IV	X	Y
(1) [a]	4.04	27.06	39.79	3.08	18.47	7.56	—	—
(2) [b]	2.27	11.28	13.84	0.80	1.07	1.13	66.32	3.29

[a] With the commercial coffee bean α-galactosidase preparation.
[b] With a crude α-galactosidase prepared from green coffee beans.

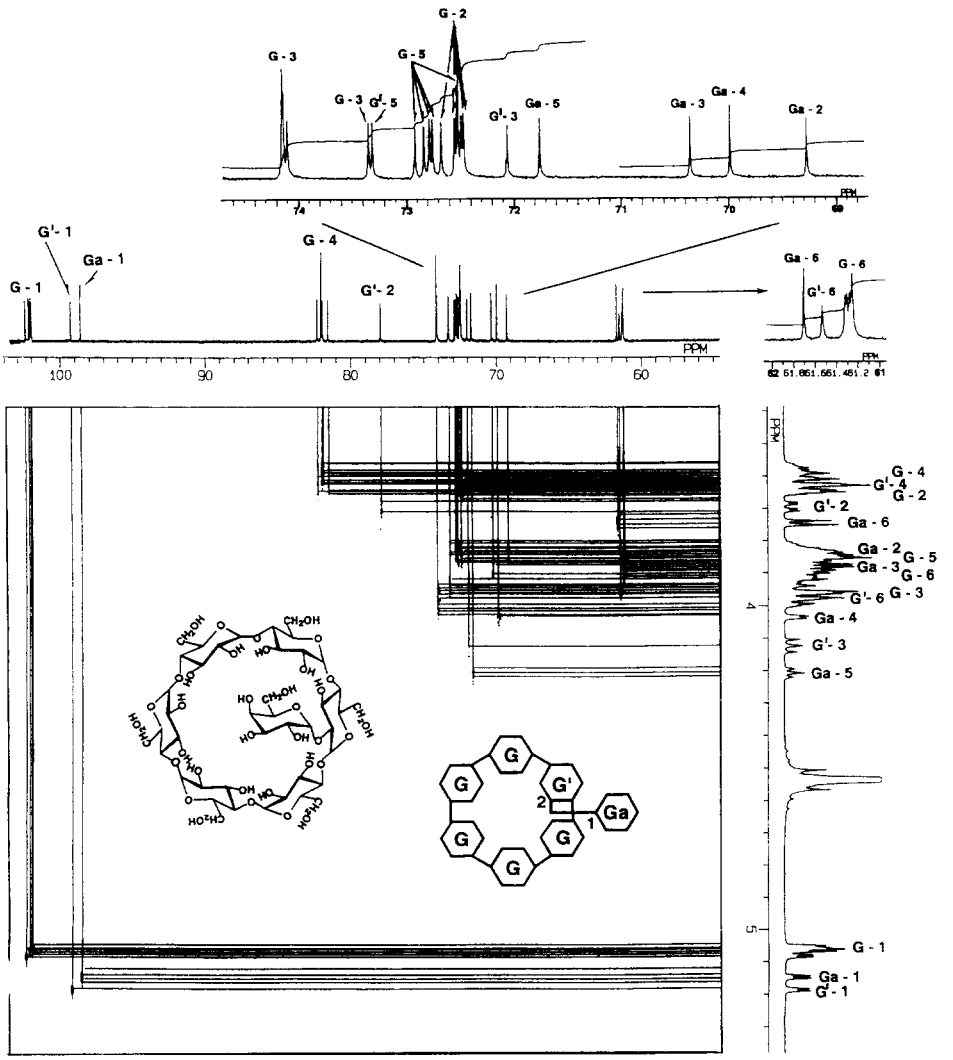

Fig. 4. $^{1}H-^{13}C$ COSY spectrum of 2-*O*-α-D-galactopyranosyl-αCD [Gal-αCD (**B**)] in D_2O at 50°C.

Table 4
The molecular ion (●) and primary fragments (○) in the FABMS spectra of digalactosyl-α and -βCDs in the negative-ion mode

Compound	m/z				Side chain
	971	1133	1295	1457	
Di-Gal-αCD					
1a–1c		○	●		digalactosyl-
2a,2b		○	●		digalactosyl-
3		○	●		digalactosyl-
4	○	○	●		galactobiosyl-
Di-Gal-βCD					
I		○	○	●	galactobiosyl-
IIa–IIc			○	●	digalactosyl-
IIIa–IIIe			○	●	digalactosyl-
IV		○	○	●	galactobiosyl-
X		○	○	●	galactobiosyl-

shift of that carbon atom, moving it downfield by 8–11 ppm [7]. The large downfield shift of one C-2 signal (G′-2) was observed in the spectra of Gal-αCD (**B**), Gal-βCD (**B**), and Gal-γCD (**B**). Consequently, the minor product (**B**) of monogalactosylation to each CD was ascertained to be 2-*O*-α-D-galactopyranosyl-CD. An additional characteristic of the ^{13}C NMR spectrum of **B** was the upfield shift of one C-1 signal of the CD ring glucose on which the galactosyl residue was attached at C-2 (β effect).

(2) Digalactosyl-CDs. FABMS studies of digalactosyl-CDs can be used to determine, not only estimates of the molecular weights, but also whether two galactosyl residues of digalactosyl-CDs link directly to the parent CD ring or form a side chain of galactobiose. Thus in the FABMS spectrum of the former in the negative-ion mode, the molecular ion peak $[M - H]^-$ and only one fragment ion peak $[M - Gal - H]^-$ should be observed, while the spectrum of the latter shows additional fragment ion peak $[M - 2Gal - H]^-$, since all these fragment ions must be formed through one cleavage of the side chain (primary fragments) [8]. In Table 4, the molecular ion and primary fragments in the FABMS spectra of digalactosyl-CDs in the negative-ion mode are summarized and their side chains are predicted. The molecular ion peak $[M - H]^-$ of the compound **Y** corresponding to peak Y in Fig. 3-[2] was observed at *m/z* 1619 together with three fragment ion peaks at *m/z* 1457, 1295, and 1133. Consequently, this compound is suggested to be a galactotriosyl-βCD.

^{13}C NMR spectroscopy of digalactosyl-CDs offered further corroborating evidence relating to their structures. The spectra of digalactosyl-CDs were similar to those of corresponding diglucosyl-CDs [8,9], and hence assignments of signals could be made by analogy. The assignments of the signals of C-6, to which galactosyl residues are attached in many cases, were confirmed by the distortionless enhancement by polarization transfer (DEPT) method [10].

Fig. 5 shows the ^{13}C NMR spectra of digalactosyl-αCD (**1c**, **2a**, **2b**, and **3**) in D_2O. In the spectrum of **1c**, each signal for galactosyl-substituted C-6 (G′-6) and C-2 (G″-2)

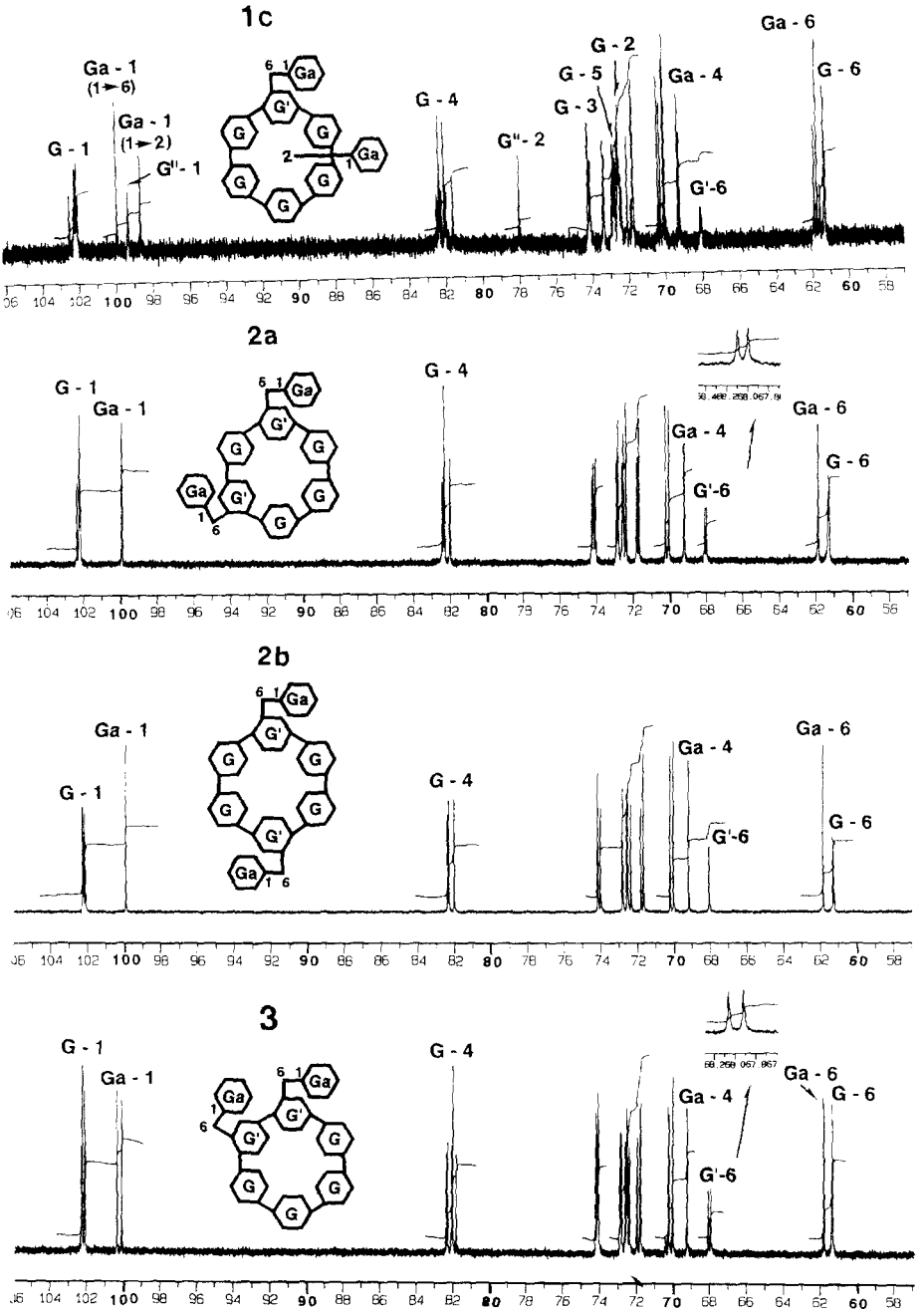

Fig. 5. ^{13}C NMR spectra of digalactosyl-αCDs (**1c**, **2a**, **2b**, and **3**) in D_2O at 50°C. G-1, -2, -3, -4, -5, and -6 are signals of C-1, -2, -3, -4, -5, and -6 atoms of the ring D-glucopyranose units. G′ and G″ are the ring D-glucopyranose units on which galactopyranosyl residue is (1 → 6)-linked and (1 → 2)-linked, respectively. Ga-1, -4, and -6 are signals of C-1, -4, and -6 atoms of the side-chain D-galactopyranose units.

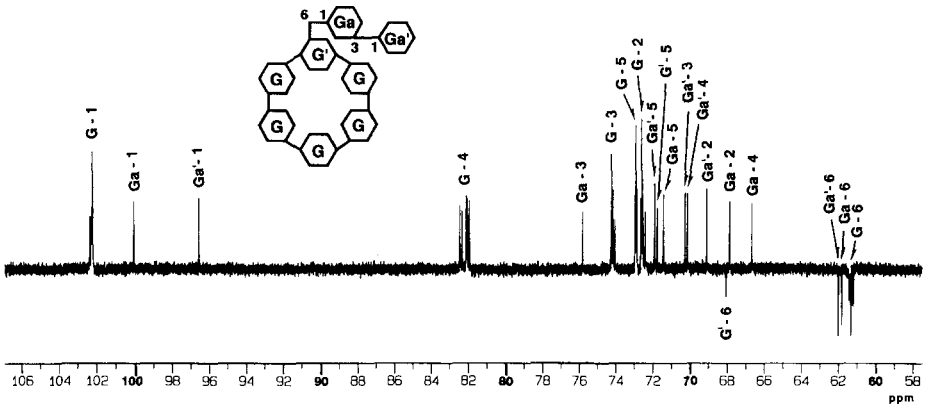

Fig. 6. ^{13}C NMR spectrum of digalactosyl-αCD (**4**) with the DEPT method in D_2O at 50°C.

shifted downfield by 6–7 ppm from the other C-6s (δ ~ 61.3) and C-2s (δ ~ 72.5), appearing at δ ~ 68 and ~ 78, respectively. The spectra of digalactosyl-αCD (**1a**) and (**1b**) were similar to that of **1c**. Therefore, these compounds were supposed to be positional isomers of $6^1,2^n$-di-*O*-(α-D-galactopyranosyl)-αCD (n = 1–6). On the other hand, two signals for C-6s (G′-6) shifted downfield were observed in the spectra of **2a**, **2b**, and **3**, and a detailed comparison of their spectra indicated the difference of magnitude of interaction between two side-chain galactose units in each molecule. These results suggest that **2a**, **2b**, and **3** are $6^1,6^3$-, $6^1,6^4$- and $6^1,6^2$-di-*O*-(α-D-galactopyranosyl)-αCDs.

Compound **4** had a very long retention time on the ODS column with methanol–water, and FABMS spectroscopy suggested a galactobiosyl-αCD. The ^{13}C NMR resonances of all carbons in the spectrum of this quite new derivative were assigned using ^1H–^1H COSY and ^1H–^{13}C COSY methods. Fig. 6 shows the ^{13}C NMR spectrum of **4** using the DEPT method. The shift of the C-6 signal (C′-6) of the αCD-ring glucose downward to δ 68 indicates a galactosyl residue substituted on the oxygen atom attached to the C-6. Moreover, one C-3 signal (Ga-3) of two side-chain galactoses appeared at δ 75.5, shifting downfield by 5.2 ppm from another C-3 signal of galactose (Ga′-3). Consequently, **4** was thought to be 6-*O*-α-(3-*O*-α-D-galactopyranosyl)-D-galactopyranosyl-αCD.

In the ^{13}C NMR spectrum of digalactosyl-βCD (**I**), one signal for the C-6 of βCD-ring glucose (G′-6, δ 68.5) and one C-6 signal of side-chain galactose (Ga-6, δ 67.2) shifted downfield from the other C-6s (G-6, δ ~ 61.2 and Ga′-6, δ 61.9) (Fig. 7). Therefore, **I** was 6-*O*-α-(6-*O*-α-D-galactopyranosyl)-D-galactopyranosyl-βCD. At 50°C broadening of the G′-6 signal was observed and suggested that free rotation of the linkage between βCD ring and galactobiose side-chain was hindered. The broadening was improved at 80°C. Although the presence of the analogous galactobiosyl-αCD in a mixture of digalactosyl-αCDs was expected, it has not been isolated yet, since it would coelute with digalactosyl-αCDs (**1s**) and attempts to separate them have been unsuccessful.

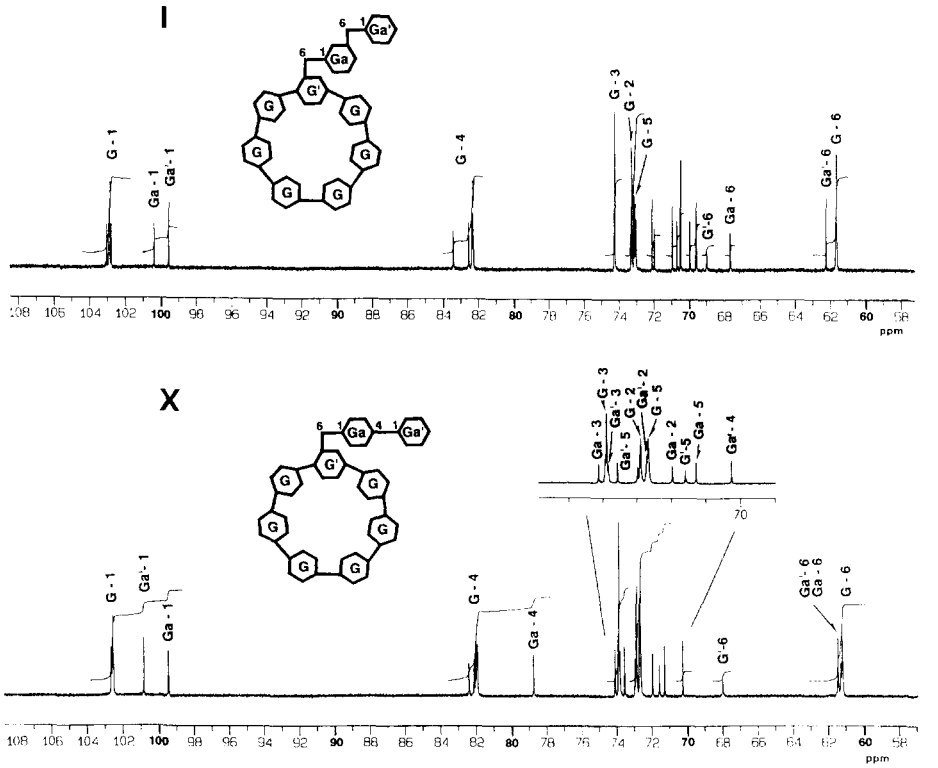

Fig. 7. ^{13}C NMR spectra of digalactosyl-βCD (**I**) in D_2O at 80°C and (**X**) in D_2O at 50°C.

The ^{13}C NMR spectra of **IIa**, **IIb**, and **IIc** were similar to those of $6^1,6^n$-di-*O*-(α-D-galactopyranosyl)-αCDs (**2a**, **2b**, and **3**). Two signals for C-6s shifted downfield (from δ ~ 61.2 to δ ~ 68.2) were observed in each spectrum, and a detailed comparison of their spectra suggest that **IIa**, **IIb**, and **IIc** are $6^1,6^3$-, $6^1,6^4$- and $6^1,6^2$-di-*O*-(α-D-galactopyranosyl)-βCDs.

The ^{13}C NMR spectrum of **III** suggested that **III** was a mixture of $6^1,2^n$-di-*O*-(α-D-galactopyranosyl)-βCDs ($n = 1$–7). The spectrum of **III** was similar to that of $6^1,2^n$-di-*O*-(α-D-galactopyranosyl)-αCD (**1c**, Fig. 5), with signals for galactosyl-substituted C-6 (G′-6) and C-2 (G″-2) shifted downfield from the other C-6s and C-2s, appearing at δ ~ 68.2 and δ 78.2–78.6, respectively. The relative intensities of G′-6 and G″-2 were 1:1, and the α-(1 → 6)- and α-(1 → 2)-linked C-1 signals of the galactose residues and the C-1 signal of the CD ring glucose that was shifted upfield by the β effect were observed at δ 100.0, 98.8–99.0 and 99.5–99.6, respectively.

Compound **IV** was similar in its elution behavior on the ODS column with methanol–water to that of a galactobiosyl-αCD (**4**) and was suggested by FABMS spectroscopy to be a galactobiosyl-βCD. Furthermore, **IV** and **4** gave similar ^{13}C NMR spectra. One C-6 signal of the CD-ring glucose and one C-3 signal of side-chain

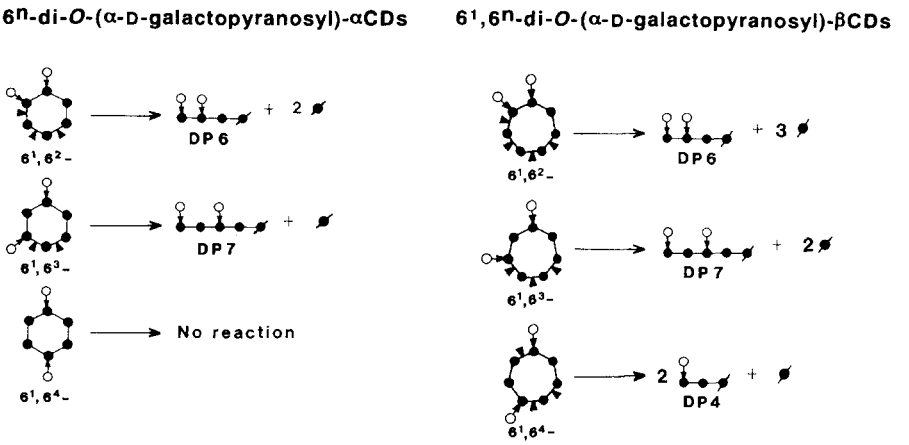

Fig. 8. Models of reaction on each three positional isomers of $6^1,6^n$-di-*O*-(α-D-galactopyranosyl)-αCD and -βCD with CGTase. Symbols: ●, glucose; ◖, glucose with reducing end; ○, galactose; ●–●, α-(1 → 4)-glucosidic linkage; ○–●, α-(1 → 6)-galactosidic linkage; ▲, point of attack.

galactose shifted downward to δ 68.2 and δ 75.5, respectively. These results suggest that **IV** is 6-*O*-α-(3-*O*-α-D-galactopyranosyl)-D-galactopyranosyl-βCD.

The main product (**X**) in a mixture of digalactosyl-βCDs (see Fig. 3-[2]) prepared by the use of a crude α-galactosidase from green coffee beans was not found in another mixture of digalactosyl-βCDs (see Fig. 3-[1]) synthesized using the commercial enzyme. In order to determine the structure of **X**, which was presumed by FABMS to be a

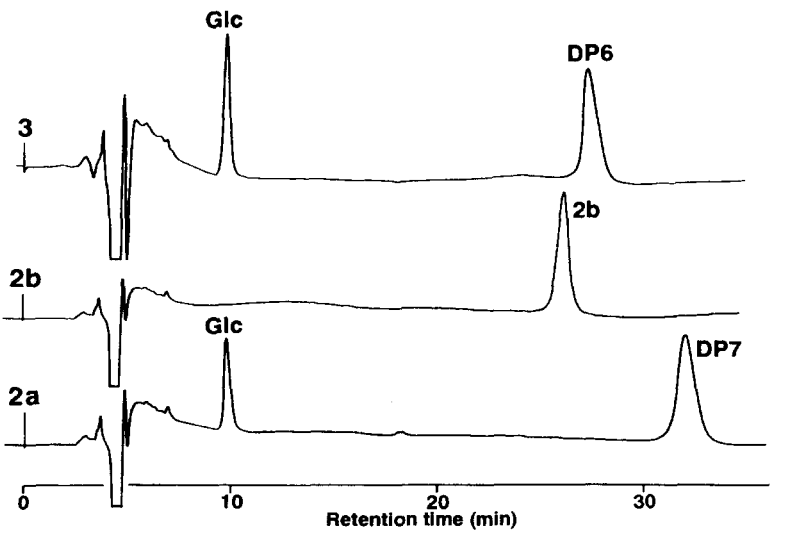

Fig. 9. Chromatograms of digestion products of **2a**, **2b**, and **3** with CGTase on a YMC-Pack Polyamine-II column (250×6 mm i.d.) with 57:43 acetonitrile–water.

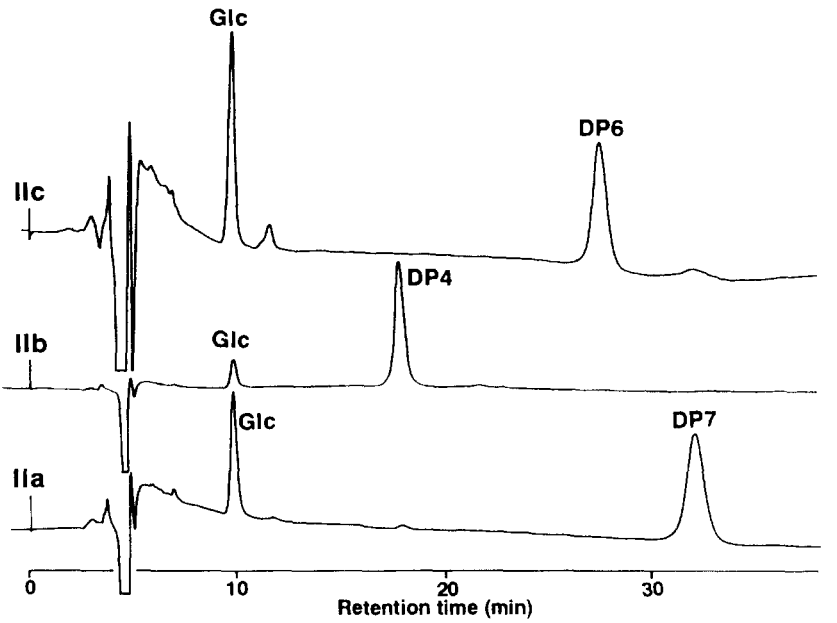

Fig. 10. Chromatograms of digestion products of **IIa**, **IIb** and **IIc** with CGTase on a YMC-Pack Polyamine-II column (250×6 mm i.d.) with 57:43 acetonitrile–water.

galactobiosyl-βCD, the ^{13}C resonances of all carbons in the spectrum of **X** were assigned using ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY methods. In the ^{13}C NMR spectrum of **X**, one C-6 signal of the βCD-ring glucose (G′-6, δ 68.0) and one C-4 signal of the side-chain galactose (Ga-4, δ 78.8) shifted downfield (Fig. 7). Consequently, **X** is 6-*O*-α-(4-*O*-α-D-galactopyranosyl)-D-galactopyranosyl-βCD. On the other hand, the commercial α-galactosidase preparation from coffee beans did not synthesize **X** even under the same reaction conditions that differed from the usual one in terms of presence of ammonium sulfate, and hence it was thought that ammonium sulfate did not influence the production of **X**, but this may be dependent on the origin of enzymes. A detailed study on this crude enzyme will be reported in the near future.

A minor product (**Y**) having the longest retention time in Fig. 3-[2] was presumed to be a galactotriosyl-βCD by FABMS. In the ^{13}C NMR spectrum of **Y**, one C-6 signal of the CD-ring glucose (δ 68.0) and two C-4 signals of side-chain galactoses (δ 78.1 and 79.2) were shifted downfield, and one α-(1 → 6)- and two α-(1 → 4)-linked C-1 signals of the galactose residues appeared at δ 99.4 and 100.7, 100.8, respectively. Consequently, **Y** has one more galactose residue substituted on the oxygen atom attached to C-4 of the nonreducing end galactose in side-chain of **X**.

Structures of each three positional isomers of 6^{1},6^{n}-di-*O*-(α-D-galactosyl)-αCD (**2a**, **2b**, and **3**) and -βCD (**IIa**, **IIb**, and **IIc**) were elucidated by analyses of fragments produced by digestion of them with CGTase. Kobayashi proposed the action mechanism of CGTase for three positional isomers of 6^{1},6^{n}-di-*O*-(α-D-glucosyl)-αCD [11]. From analogy with these, possible fragments from positional isomers of 6^{1},6^{n}-di-*O*-(α-D-

galactosyl)-αCD and -βCD are summarized in Fig. 8. These fragments have different degree of polymerizations (dps) and, therefore, structural analyses of those positional isomers could be solved by determination of the dps of fragment oligosaccharides using HPLC on an amino-bonded column and FABMS. Fig. 9 shows chromatograms of the hydrolysates of **2a**, **2b**, and **3**. The reaction product from **2b** was identical with the starting material. Hydrolysates of **2a** and **3** were isolated, and their molecular weights were determined by FABMS: **2a**, 1152; **3**, 990. These results suggested that **2a**, **2b**, and **3** were $6^1,6^3$-, $6^1,6^4$-, and $6^1,6^2$-di-*O*-(α-D-galactopyranosyl)-αCDs. In a similar manner the chromatographic behavior of hydrolysates of **IIa**, **IIb** and **IIc** on an amino-bonded column (Fig. 10) and their molecular weights determined by FABMS (**IIa**, 1152; **IIb**, 666; **IIc**, 990) indicated that **IIa**, **IIb** and **IIc** were $6^1,6^3$-, $6^1,6^4$-, and $6^1,6^2$-di-*O*-(α-D-galactopyranosyl)-βCDs. These results were in accord with expectations based on the ^{13}C NMR data.

4. Conclusions

Coffee bean α-galactosidase catalyzes the transgalactosylation from melibiose mainly to the C-6 position, and also, to a limited extent, to the C-2 position of the CD-ring glucose. When the transgalactosylation was carried out with a higher concentration of melibiose for an extended period, digalactosylated CDs were produced to an extent of 10% of the monogalactosyl-CDs. Although in the digalactosylation the second galactosyl residue transferred mainly to the C-6 of the CD-ring glucose, transgalactosylation to the C-6 or the C-3 of side-chain galactose was also observed. On the other hand, digalactosylation of βCD with a crude α-galactosidase prepared from green coffee beans occurred on the C-4 of side-chain galactose. We presume that the difference derives from the origin of enzyme.

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