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Isolation by HPLC of the positional isomers of $6^1,2^n$-di-*O*-α-D-galactopyranosylcyclomaltohexaose ($n = 1–6$) and determination of their structures by enzymatic degradation

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Abstract

The six positional isomers of $6^1,2^n$-di-*O*-α-D-galactopyranosylcyclomaltohexaose (α-cyclodextrin, αCD, $n = 1–6$), which were produced from αCD and melibiose by transgalactosylation with coffee bean α-galactosidase, were isolated by HPLC on a reversed-phase column and a graphitized carbon column. The structures of those isomers were elucidated by LC–MS analyses of enzymatic degradation products with cyclomaltodextrin glucanotransferase and α-galactosidase. © 1997 Elsevier Science Ltd. All rights reserved.

Keywords: Cyclomaltohexaose (αCD); Heterogeneous doubly branched; Enzymatic degradation; HPLC; LC–MS; Positional isomer, separation; Structural analysis

1. Introduction

Recently, heterogeneous branched cyclomaltooligosaccharides (cyclodextrins, CDs) have been expected to be useful in new applications that are different from those of conventional CDs and homogeneous branched CDs. The separation and structural analysis of the several galactosyl-CDs obtained by transgalactosylation with α-galactosidase from coffee bean (EC 3.2.1.22) have been reported thus far [1–3]. Transgalactosylation products contained abundant mono-galactosylated CDs and small amounts of di-galactosylated CDs [3]. In the case of the transgalactosylation of αCD, mono-galactosylated products were mainly 6-*O*-α-D-galactopyranosyl-αCD, and 2-*O*-α-D-galactopyranosyl-αCD as a minor component [3]. It was also proved that di-galactosylated products having two galactosyl residues directly linked to αCD ring consisted of two different types of positional isomers of $6^1,6^m$-di-*O*-α-D-galactopyranosyl-αCDs [$6^1,6^m$-$(Gal)_2$-αCDs, $m = 2–4$] and $6^1,2^n$-di-*O*-α-D-

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galactopyranosyl-αCDs [$6^1,2^n$-$(Gal)_2$-αCDs, $n = 1$–6] [3]. The structures of three positional isomers of $6^1,6^m$-$(Gal)_2$-αCDs were easily elucidated from the results of HPLC and FABMS of the branched oligosaccharides produced by degradation with cyclomaltodextrin glucanotransferase (CGTase) (EC 2.4.1.19) [3]. However, the separation and the structural analysis of $6^1,2^n$-$(Gal)_2$-αCDs consisting of six positional isomers have not yet been achieved.

We report here the separation by HPLC of $6^1,2^n$-$(Gal)_2$-αCDs and their structural determination by analysis of enzymatically degradated products using LC–MS as a new tool.

2. Experimental

Materials.—According to the method described in the previous paper [2], a mixture of the transgalactosylated αCDs was prepared from αCD and melibiose with coffee bean α-galactosidase. CGTase (1200 U/mL) [4] from *Bacillus stearothermophilus* and α-galactosidase (60 U/mL) [5] from *Candida guilliermondii* were each prepared and purified by the previously reported method. All reagents were of analytical grade. Reagent-grade organic solvents used for chromatography were dried and freshly distilled before use. Water used in solvent preparations was distilled, deionized, and redistilled.

General methods.—HPLC was performed with a Jasco 980 PU pump, a Rheodyne 7125 injector, and a Shodex RI-71 monitor (Showa Denko). HPLC analyses at constant temperature were conducted with an SSC 3510C column oven (Senshu Scientific). The columns used were a YMC-Pack A-323-3 ODS (250 × 10 mm i.d.) (YMC), a Hypercarb (100 × 4.6 mm i.d.) (Shandon) and a Carbonex (150 × 10 mm i.d.) (TONEN) for the analysis and purification of $6^1,2^n$-$(Gal)_2$-αCDs, and a YMC-Pack Polyamine-II (150 × 4.6 mm i.d.) for the analysis of their enzymatically degradated products.

LC–MS was carried out using a Hewlett–Packard HP1050 series Model HP79852A pump interfaced to a Finnigan TSQ-7000 triple-stage quadrupole mass spectrometer (Finnigan MAT Instruments, San Jose, CA) fitted with the Finnigan electrospray ionization (ESI) interface. The mass spectrometer was operated in the positive-ion mode; the ESI voltage was set to 4.5 kV, and the ESI current was 0.34 μA. The capillary temperature was 230 °C. The pressure of the sheath gas was 70 psi, and the auxiliary gas was 15 u. Total ion monitoring was done by scanning covered the range m/z 800–1500 with a scan rate of 2 s/scan.

FABMS was performed in the negative-ion mode using a JEOL JMS-DX 303 mass spectrometer with Xe 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.

^{13}C NMR spectra (125.65 MHz) were recorded for 2–3% solutions in D_2O at 50 °C, using 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).

Structural analyses of positional isomers by enzymatic degradation.—Each sample (0.2 mg) of $6^1,2^n$-$(Gal)_2$-αCDs (**A**, **B**, **C**, **E**, **F1**, and **F2**) in 70 μL of 10 mM acetate buffer (pH 6.0) was individually incubated with CGTase (20 μL, 24 U) at 40 °C for 24 h. Then the enzyme was inactivated by placing it in a boiling water bath for 10 min. Each hydrolysate with CGTase was analyzed by HPLC and LC–MS. An additional amount of α-galactosidase was added to the reaction mixture, if necessary, and the mixture was incubated at 40 °C for 6–24 h. At the end of this time, the enzyme was inactivated. The hydrolysates were analyzed by HPLC and LC–MS.

3. Results and discussion

Separation.—Fig. 1 shows the chromatogram on an ODS column of a mixture of di-galactosylated αCDs produced from αCD and melibiose by transgalactosylation with coffee bean α-galactosidase. The structures of products corresponding to peaks 2–4 were already elucidated to be a mixture of $6^1,6^3$- and $6^1,6^4$-$(Gal)_2$-αCDs, $6^1,6^2$-$(Gal)_2$-αCD, and 6-*O*-α-(3-*O*-α-D-galactopyranosyl)-D-galactopyranosyl-αCD, respectively [3]. It was presumed that fraction 1 was comprised of several components that corresponded to $6^1,2^n$-$(Gal)_2$-αCDs as determined by ^{13}C NMR spectrometry and by FABMS [3]. Therefore, a larger ODS column packed with the same stationary phase, YMC-Pack A-323-3 (250 × 10 mm i.d.) and eluted with a methanol–water system was used to purify each component of fraction 1 (Fig. 2). Although six components (**A**–**F**) were separated on this ODS column, it was predicted that **D** was not a member of a homologous series of $6^1,2^n$-$(Gal)_2$-αCDs because the elution behavior of **D** on this system was different from those of other components.

F was separated into two components (**F1** and **F2**)

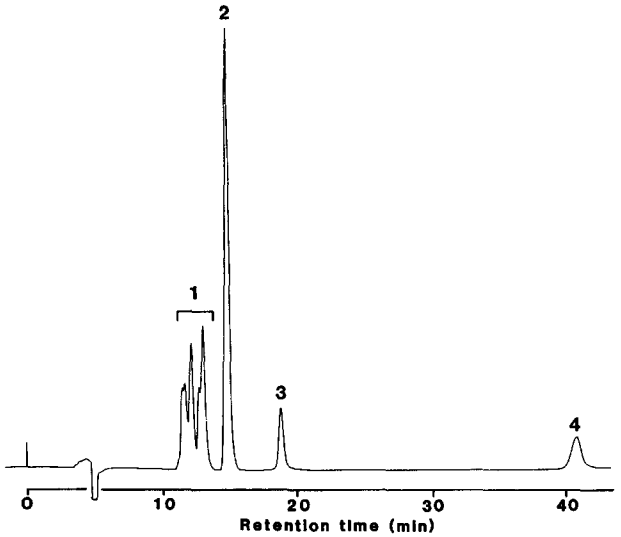

Fig. 1. Elution profile of di-galactosylated αCDs. 1: mixture of $6^1,2^n$-$(Gal)_2$-αCDs, 2: mixture of $6^1,6^3$- and $6^1,6^4$-di-*O*-α-D-galactopyranosyl-αCDs, 3: $6^1,6^2$-di-*O*-α-D-galactopyranosyl-αCD, 4: 6-*O*-α-(3-*O*-α-D-galactopyranosyl)-D-galactopyranosyl-αCD. Chromatographic conditions: column, YMC-Pack A-312-3 (150×6 mm i.d.); eluent, 4:96 CH_3OH–H_2O; flow rate, 0.7 mL/min; temperature, 30 °C.

on a graphitized carbon column, Hypercarb (100 × 4.6 mm i.d.), with an acetonitrile–water system (the insert in Fig. 2). All components were isolated by HPLC using both an ODS and a larger carbon column, a Carbonex (100 × 10 mm i.d.).

FABMS and ^{13}C NMR.—In the negative-ion FABMS spectra of seven compounds (**A**–**F2**), both a molecular ion $[M - H]^-$ peak at *m/z* 1295 and fragment ion $[M - Gal - H]^-$ peak at *m/z* 1133, which formed through cleavage of one galactosyl side chain, were observed. Moreover, the characteristic fragment ion $[M - 2Gal - H]^-$ peak at *m/z* 971, which formed through cleavage of one galactobiosyl side chain, was observed in the spectrum of **D**, and this spectrum was similar to that of 6-*O*-α-(3-*O*-α-D-galactopyranosyl)-D-galactopyranosyl-αCD (peak 4 in Fig. 1), though the fragment ion *m/z* 971 was not detected in the spectra of other compounds. These phenomena suggest that all compounds are made up of an αCD ring and two galactosyl residues, **D** is a mono-branched αCD in which one galactobiosyl side chain is attached directly to the αCD ring, and the others are doubly branched αCDs having two galactosyl side chains.

The structure of **D** was revealed by ^{13}C NMR spectroscopy. Assignments of signals were made by comparison with those in the spectra of 6-*O*-α-D-galactopyranosyl-αCD [2] and 6-*O*-α-(6-*O*-α-D-galactopyranosyl)-D-galactopyranosyl-βCD [3]. It is known that a substituent on the oxygen atom attached to any carbon atom of the sugar moiety affects the chemical shift of the carbon atom moving it downfield by 8–11 ppm [6]. In the ^{13}C NMR spectrum of **D**, four kinds of signals for the C-6 were confirmed by the distortionless enhancement by polarization transfer (DEPT) method [7]. The signals for the C-6s of the αCD ring of five glucoses (δ 61.2–61.3), which have only α-(1 → 4)-glucosidic linkages, and one C-6 signal of the terminal galactosyl side chain (δ 61.9), and the two large downfield-shifted C-6 signals (δ 67.9 and 67.3) were observed. The downfield-shift to δ 67.9 of the C-6 signal of the αCD ring glucose indicated that one galactobiosyl residue was substituted on the oxygen atom attached to the C-6. Similarly another downfield-shifted C-6 signal (δ 67.3) of galactose in galactobiose suggested that the terminal galactosyl residue was substituted at C-6 of galactosyl side chain. The relative signal intensities of the αCD ring C-6s (δ ~ 61.3), the terminal galactosyl side chain C-6 (δ 61.9), another galactosyl side chain C-6 (δ 67.3), and the αCD ring branch-point C-6 (δ 67.9) were 5:1:1:1. Moreover, the characteristic two C-1 signals of side-chain galactobiose appeared at higher field than those of the C-1 signals of the αCD ring glucoses (δ 102.2–102.4): α-(1 → 6)-linked C-1 signal of the terminal galactosyl residue and the C-1 signal of the another galactosyl side chain which α-(1 → 6)-linked to αCD ring were observed at δ 99.1 and 99.7, respectively. The relative signal intensities of the αCD ring C-1s and two

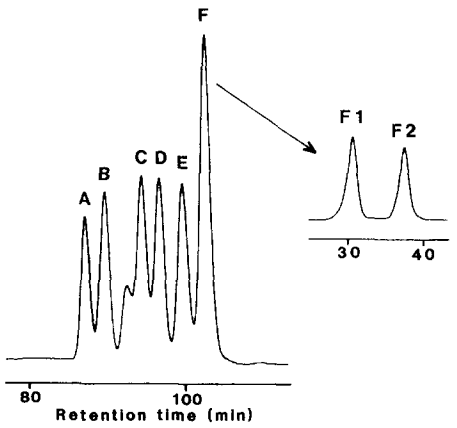

Fig. 2. Re-chromatograms of fraction 1. Chromatographic conditions: column, YMC-Pack A-323-3 (250×10 mm i.d.); eluent, 3:97 CH_3OH–H_2O; flow rate, 0.7 mL/min; temperature, 30 °C. Special conditions for separation of **F1** and **F2**: column, Hypercarb (100×4.6 mm i.d.); eluent, 10:90 CH_3CN–H_2O; flow rate; 0.5 mL/min; temperature, 30 °C.

kinds of galactose C-1s were 6:1:1. Consequently, **D** was thought to be 6-*O*-α-(6-*O*-α-D-galactopyranosyl)-D-galactopyranosyl-αCD.

Structural analysis.—We reported in the preceding paper [8] that the structures of the six positional isomers of 2^n-*O*-α-D-galactopyranosyl-6^1-*O*-α-D-glucopyranosyl-αCDs (2^n-Gal-6^1-Glc-αCDs, $n = 1$–6) were elucidated by HPLC and FABMS analyses of their digestion products with a combination of CGTase and glucoamylase (EC 3.2.1.3). It is easily presumed that a mode of action of CGTase for $6^1,2^n$-$(Gal)_2$-αCDs is the same as that for 2^n-Gal-6^1-Glc-αCDs. The models of reaction of $6^1,2^n$-$(Gal)_2$-αCDs with CGTase and α-galactosidase are shown in Fig. 3. Fig. 4 shows the chromatograms by RI detection and the mass chromatograms of the enzymatic degradation products of **A**–**F2** with CGTase. In the LC–MS analysis, the mass chromatograms at special *m/z* 1008, 1170, 1008, 1170, 1332, and 1314 for the enzymatic digests of **A**, **B**, **C**, **E**, and **F1** (**1**–**5**), and **F2**, respectively, were monitored as adduct ions with ammonium ion $[M + NH_4]^+$ in the positive-ion ESI mode. **F2** remained intact by CGTase, and the adduct ion could be observed at *m/z* 1314. On the other hand, no glucose and only **5**, which was formed by ring opening of $6^1,2^n$-$(Gal)_2$-αCD, was detected in the degradation products of **F1**. The degree of polymerization (dp) of **5** was 8, because the adduct ion was observed at *m/z* 1332. Accordingly, it was determined that **F1** and **F2** corresponded to $6^1,2^3$- and $6^1,2^4$-di-*O*-α-D-galactopyranosyl-αCDs, respectively. The degradation products (**1** and **3**) of the same dp — dp 6 (*m/z* 1008) — were obtained from **A** and **C**. Similarly, oligosaccharides (**2** and **4**)

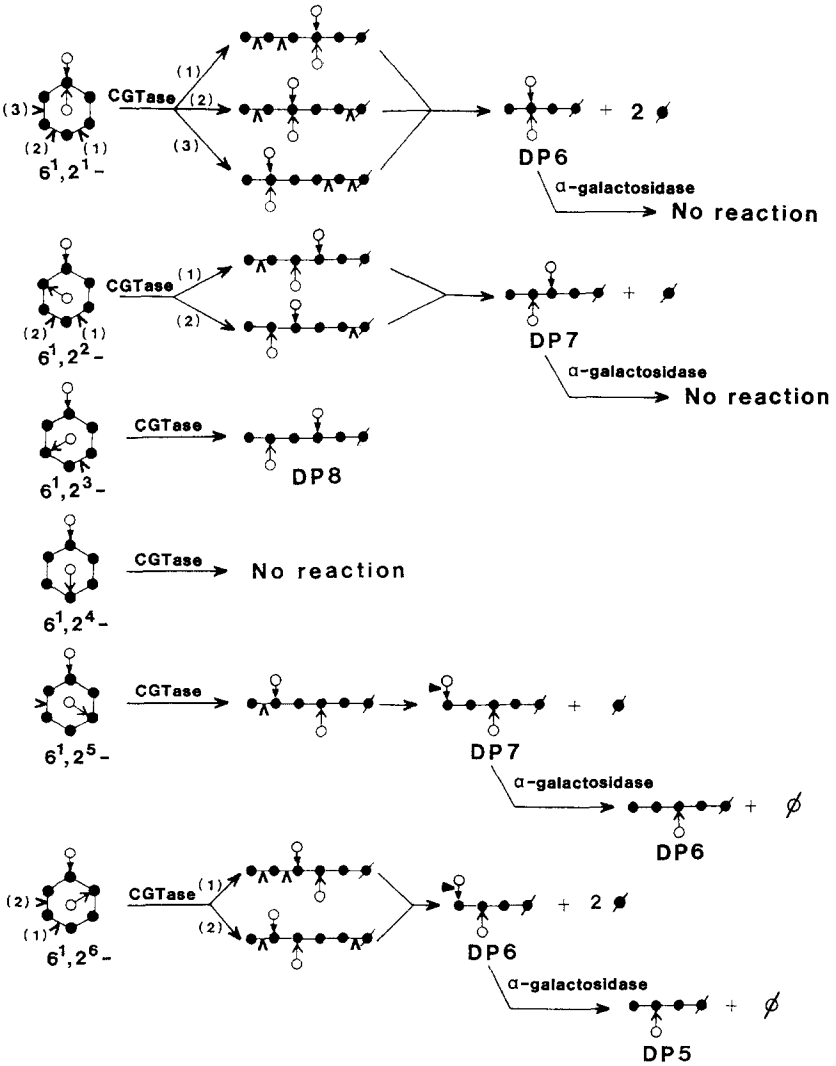

Fig. 3. Models of reaction on six positional isomers of $6^1,2^n$-$(Gal)_2$-αCDs with CGTase and α-galactosidase. Symbols: ●, glucose; ◉, glucose with reducing end; ○, galactose; ⊘, galactose with reducing end; ●–●, α-(1 → 4)-glucosidic linkage; ⚷, α-(1 → 6)-galactosidic linkage; ⚷, α-(1 → 2)-galactosidic linkage; ∧, attack point of CGTase; ▲, attack point of α-galactosidase.

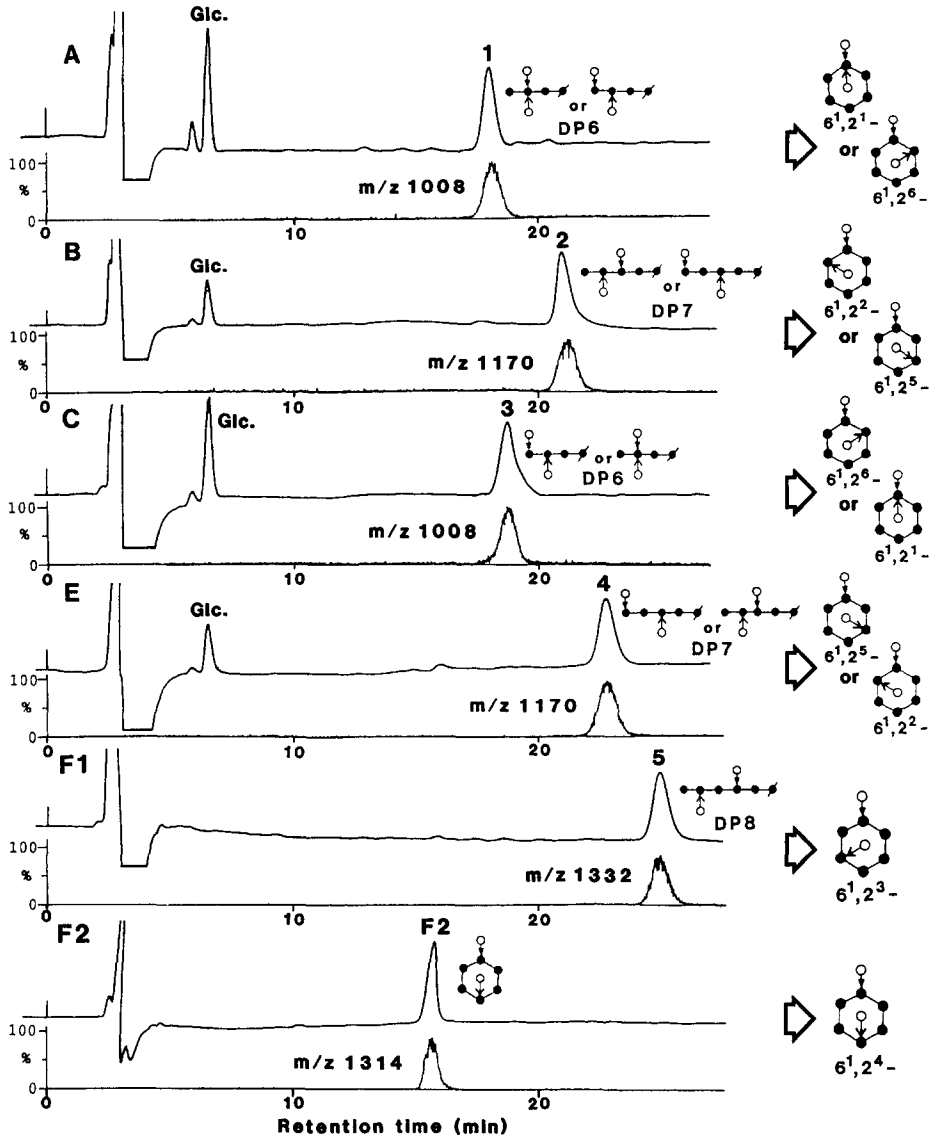

Fig. 4. Chromatograms of degradation products from **A**, **B**, **C**, **E**, **F1**, and **F2** with CGTase and mass chromatograms of their ammonium adducts. Chromatographic conditions: column, YMC-Pack Polyamine-II (150 × 4.6 mm i.d.); eluent, 57:43 CH_3CN–10 mM CH_3COONH_4; flow rate, 0.5 mL/min; temperature, 30 °C. Each upper chromatogram was detected by RI.

of dp 7 (*m/z* 1170) were detected in the cases of **B** and **E**. For the distinction between **1** and **3**, and **2** and **4**, respectively, they were further treated with α-galactosidase (Fig. 5). CGTase digests of **A** and **B** (**1** and **2**) were unchanged with α-galactosidase, while **3** and **4** obtained from **C** and **E** were decomposed to branched oligosaccharides **6** (dp 5; *m/z* 846) and **7** (dp 6; *m/z* 1008), respectively, each of which lost one galactosyl residue. This result means that **3** and **4** have the nonreducing terminal of an α-(1 → 6)-galactosidic linkage that can be hydrolyzed with α-galactosidase. Therefore, it was established that **A**, **B**, **C**, and **E** were $6^1,2^1$-, $6^1,2^2$-, $6^1,2^6$-, and $6^1,2^5$-di-*O*-α-D-galactopyranosyl-αCDs, respectively.

If we look more carefully at the chromatograms of the HPLCs, we perceive interesting chromatographic behavior of the digests of $6^1,2^n$-$(Gal)_2$-αCDs with CGTase and α-galactosidase on an amino-bonded silica gel column, YMC-Pack Polyamine-II with an acetonitrile–water system. In the previous paper [8], we reported that the oligosaccharides having a glucosyl side chain on the nonreducing end was eluted earlier on YMC-Pack Polyamine-II column than the isomer having a galactosyl side chain on the nonreducing end in the same dp of branched oligosaccharides. Also in this experiment, the same tendency was observed. That is to say, **3** having a galactosyl side chain on the nonreducing end was eluted later than **1**

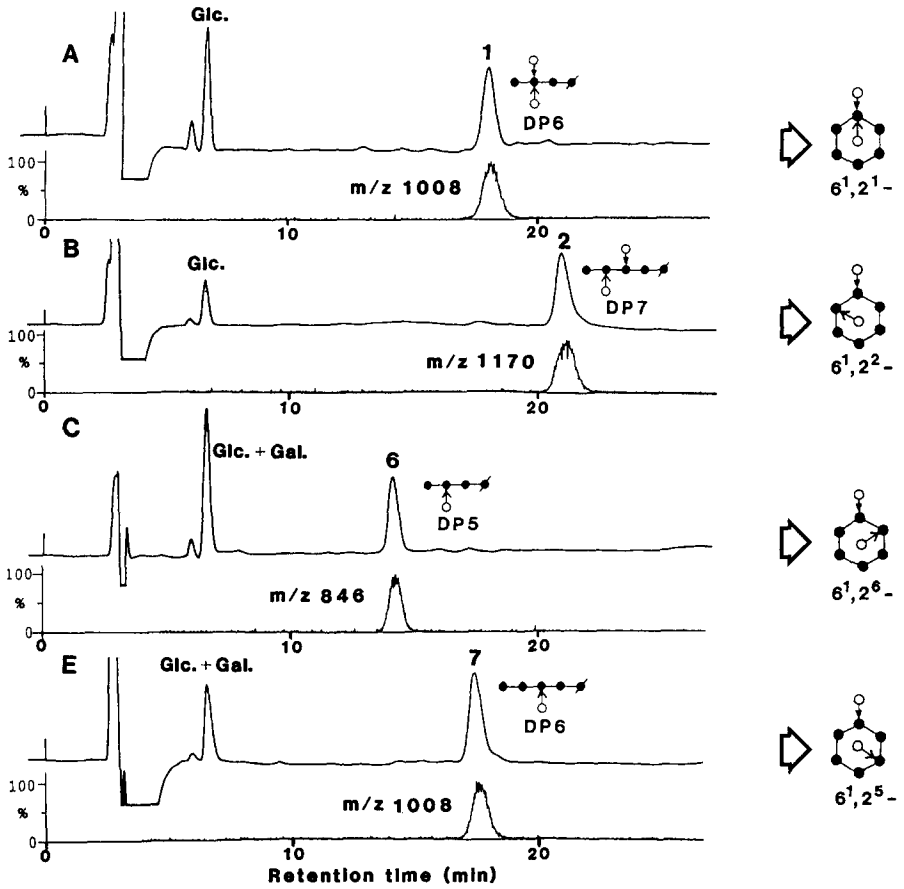

Fig. 5. Chromatograms of α-galactosidase digests of **1**, **2**, **3**, and **4** obtained from **A**, **B**, **C**, and **E** and msss chromatograms of their ammonium adducts. Chromatographic conditions as in Fig. 4.

having no galactosyl side chain on the nonreducing end, and similarly **4** was eluted later than **2**. We plan to describe in detail the chromatographic behavior of branched oligosaccharides having several different types of side chains in the near future.

4. Conclusions

The six positional isomers of $6^1,2^n$-$(Gal)_2$-αCD in the transgalactosylated products of αCD were isolated by HPLC using an ODS and a graphitized carbon column. The $6^1,2^3$- and $6^1,2^4$-isomers were not separated on an ODS column using a methanol–water system, while they were eluted as the $6^1,2^3$- and $6^1,2^4$-isomers in that order on a graphitized carbon column with an acetonitrile–water system. The structures of all isomers were elucidated by LC–MS analysis of the dp's of the branched oligosaccharides produced by enzymatic degradation with CGTase and α-galactosidase. In the first place, CGTase cleaves αCD ring of $6^1,2^n$-$(Gal)_2$-αCDs, then decomposes until the several heterogeneous branched oligosaccharides. The structures of $6^1,2^3$- and $6^1,2^4$-$(Gal)_2$-αCDs were easily determined, since the $6^1,2^4$-isomer remained intact with CGTase, and the $6^1,2^3$-isomer produced the branched oligosaccharide having the largest dp (8) among these isomers. From the $6^1,2^1$- and $6^1,2^6$-isomers, the same dp of 6 for branched oligosaccharides, in which two galactosyl residues were branched on different positions of maltotetraose, were obtained. Similarly the same dp of 7 for branched oligosaccharides were detected in hydrolyzates of $6^1,2^2$- and $6^1,2^5$-isomers. Further using α-galactosidase, these branched oligosaccharides having the same dp were distinguished. In the cases of both $6^1,2^5$- and $6^1,2^6$-isomers, the α-(1 → 6)-galactosidic linkage on the nonreducing end of the branched oligosaccharides produced with CGTase, was hydrolyzed with α-galactosidase, and resulted in conversion into dp 6 and dp 5 of the branched oligosaccharides, respectively, while the branched

oligosaccharides, which were produced from $6^1,2^1$- and $6^1,2^2$-isomers, were not altered by this enzyme. LC–MS analysis could be performed by the use of very small amounts of sample and required a short analysis time. Therefore, it was shown to be an extremely powerful tool for elucidation of structures of the positional isomers on the basis of the dp of their enzymatic degradation products.

In addition 6-*O*-α-(6-*O*-α-D-galactopyranosyl)-D-galactopyranosyl-αCD, which was expected, but not found in the previous paper [3], was isolated along with the $6^1,2^n$-$(Gal)_2$-αCDs.

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References

[1] S. Kitahata, K. Hara, K. Fujita, N. Kuwahara, and K. Koizumi, *Biosci. Biotech. Biochem.*, 56 (1992) 1518–1519.
[2] K. Hara, K. Fujita, N. Kuwahara, T. Tanimoto, H. Hashimoto, K. Koizumi, and S. Kitahata, *Biosci. Biotech. Biochem.*, 58 (1994) 652–659.
[3] K. Koizumi, T. Tanimoto, Y. Okada, K. Hara, K. Fujita, H. Hashimoto, and S. Kitahata, *Carbohydr. Res.*, 278 (1995) 129–142.
[4] S. Kitahata and S. Okada, *J. Jap. Soc. Starch Sci.*, 29 (1982) 7–12.
[5] H. Hashimoto, C. Katayama, M. Goto, and S. Kitahata, *Biosci. Biotech. Biochem.*, 57 (1993) 372–378.
[6] T. Usui, N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama, and S. Seto, *J. Chem. Soc., Perkin Trans. 1*, (1973) 2425–2432.
[7] D.M. Doddrell and D.T. Pegg, *J. Am. Chem. Soc.*, 102 (1980) 6388–6390.
[8] Y. Okada, K. Koizumi, and S. Kitahata, *Carbohydr. Res.*, 287 (1996) 213–223.