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Isolation by HPLC of the positional isomers of heterogeneous doubly branched cyclomaltohexaose having one α-D-galactosyl and one α-D-glucosyl side chain and determination of their structures by enzymatic degradation

Yasuyo Okada [a], Kyoko Koizumi [a,*], Sumio Kitahata [b]

[a] *School of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Koshien Kyuban-cho, Nishinomiya, 663, Japan*

[b] *Osaka Municipal Technical Research Institute, 1-6-50, Morinomiya, Jyoto-ku, Osaka, 536, Japan*

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Abstract

Each of the six and five positional isomers of 2^n- and 6^n-*O*-α-D-galactopyranosyl-6^1-*O*-α-D-glucopyranosyl-cyclomaltohexaose (α-cyclodextrin, αCD; 2^n: $n = 1–6$, 6^n: $n = 2–6$), which were produced from 6-*O*-α-D-glucopyranosyl-αCD and melibiose by transgalactosylation with coffee bean α-galactosidase, were isolated by high-performance liquid chromatography (HPLC) on a reversed-phase column and a graphitized carbon column. The structures of those isomers were elucidated by analyses of enzymatic degradation products with cyclomaltodextrin glucanotransferase and glucoamylase. © 1996 Elsevier Science Ltd.

Keywords: Cyclomaltohexaose (αCD), heterogeneous doubly branched; Enzymatic degradation; HPLC; Positional isomer, separation; Structural analysis

1. Introduction

Recently, Hara et al. [1] enzymatically synthesized a new type of heterogeneous branched cyclomalto-oligosaccharide (cyclodextrin, CD), that was designed as a drug delivery system for pharmaceuticals. Among these compounds were various kinds of

* Corresponding author.

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heterogeneous branched αCDs produced from 6-*O*-α-D-glucopyranosylcyclomaltohexaose (Glc-αCD) and melibiose by transgalactosylation with coffee bean α-galactosidase (EC 3.2.1.22). The main products of the transgalactosylation were heterogeneous doubly branched αCDs having one galactosyl side chain linked directly to the CD ring of Glc-αCD by an α-(1 → 6)-linkage, namely, 6^n-*O*-α-D-galactopyranosyl-6^1-*O*-α-D-glucopyranosyl-αCD (6^n-Gal-6^1-Glc-αCD, $n = 2–6$). The heretofore unprecedented heterogeneous doubly branched αCDs having one galactosyl side chain α-(1 → 2)-linked to the CD ring, that is, 2^n-*O*-α-D-galactopyranosyl-6^1-*O*-α-D-glucopyranosyl-αCD (2^n-Gal-6^1-Glc-αCD, $n = 1–6$) were also found in the transgalactosylated products. These unique derivatives have five and six positional isomers, respectively, and it is very difficult to prove the structures of these isomers by chemical synthesis and spectroscopic means. Therefore, we set out to elucidate the structures of the positional isomers using enzymatic degradation methods.

This paper deals with the separation of each of the positional isomers of two different types of the heterogeneous doubly branched αCDs (Gal-Glc-αCDs) and their structural analysis by enzymatic methods.

2. Experimental

Materials.—A mixture of the heterogeneous branched αCDs was donated by Ensuiko Sugar Refining Co., Ltd. (Yokohama, Japan). Cyclomaltodextrin glucanotransferase (CGTase) (EC 2.4.1.19) (1200 U/mL) was prepared and purified by the previously reported method [2]. A crystalline glucoamylase (EC 3.2.1.3) (37.8 U/mg) and coffee bean α-galactosidase (10.8 U/mg) were obtained from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan) and Sigma, respectively. 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 Waters U6K universal injector, and a Shodex RI-71 (Showa Denko) refractive index monitor. HPLC analyses at constant temperature were conducted with an SSC 3510C column oven (Senshu Scientific Co.). The columns used were a YMC-Pack A-312-3 ODS (150 × 6 mm i.d.) (YMC) for the analysis, a YMC-Pack A-323-3 ODS (250 × 10 mm i.d.) for the preparation, a YMC-Pack Polyamine-II (250 × 6 mm i.d.) and a Carbonex (150 × 10 mm i.d.) (TONEN) for both purposes.

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 by enzymatic degradation.—The each sample (0.2 mg) of 2^n-Gal-6^1-Glc-αCDs (**Ia**, **Ib**, **Ic**, **Id**, **Ie**, and **If**) and 6^n-Gal-6^1-Glc-αCDs (**IIa**, **IIb–c**, **IId**, and **IIe**) in 70 μL of 10 mM acetate buffer (pH 6.0) was individually incubated

with CGTase (20 μL, 24 U) and crystalline glucoamylase (0.1 mg, 3.78 U) at 40 °C for 24 h. The enzymes were inactivated at the end of this time by placing them in a boiling-water bath for 10 min. Each hydrolysate with enzymatic degradation was analyzed by HPLC on an amino-bonded silica gel column, YMC-Pack Polyamine-II and by FABMS. Degradation products (0.1 mg each) purified by HPLC and α-galactosidase (3 μL) in 70 μL of 10 mM acetate buffer (pH 6.0) were incubated at 40 °C for 2 h, at which time the enzyme was inactivated, and then the reaction mixture was analyzed by HPLC.

3. Results and discussion

Separation.—Fig. 1 is the chromatogram of the heterogeneous branched αCDs produced from Glc-αCD and melibiose by transgalactosylation with coffee bean α-galactosidase. It had already been shown that peak A corresponds to 6-*O*-α-(6-*O*-α-D-galactopyranosyl)-D-glucopyranosyl-αCD, and that peaks D and E contain 2^n-Gal-6^1-Glc-αCDs and 6^n-Gal-6^1-Glc-αCDs, respectively, by ^{13}C NMR spectrometry and by FABMS [1]. 2^n-Gal-6^1-Glc-αCDs and 6^n-Gal-6^1-Glc-αCDs each possess of a number of positional isomers, theoretically five and six isomers for 6^n- and 2^n-Gal-6^1-Glc-αCDs, respectively. However, the structures of these positional isomers have not yet been

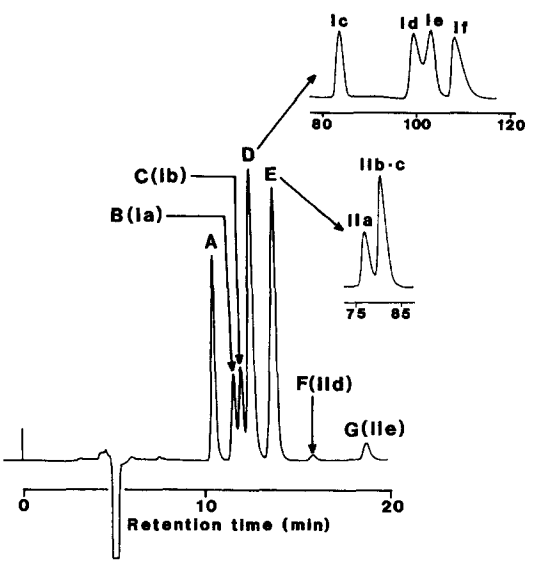

Fig. 1. Chromatograms of heterogeneous branched αCDs. A: 6-*O*-α-(6-*O*-α-D-galactopyranosyl)-D-glucopyranosyl-αCD. Chromatographic conditions: column, YMC-Pack A-312-3 (150×6 mm i.d.); eluent, 5:95 CH_3OH–H_2O; flow rate, 0.7 mL/min; temperature, 30 °C. Special conditions for separation of each isomers of D and E: column, Carbonex (150×10 mm i.d.); eluent, 10:90 CH_3CN–H_2O for D, 9:91 CH_3CN–H_2O for E; flow rate, 0.8 mL/min; temperature, 30 °C.

elucidated. Consequently, all peaks were fractionated into seven fractions (A–G), and fractions B–G were examined in further detail by HPLC under several analytical conditions. As a result, it became apparent that fractions D and E could be separated, respectively, into four peaks (**Ic**, **Id**, **Ie**, and **If**) and two peaks (**IIa** and **IIb–c**) on a graphitized carbon column (the inserts in Fig. 1). Although ^{13}C NMR spectroscopy showed the presence of two components in the latter peak of fraction E, their separation was unsuccessful.

FABMS.—In the negative-ion FABMS spectra of every Gal-Glc-αCDs, a molecular ion m/z 1295 and a fragment ion m/z 1133, which formed through cleavage of either one glucosyl or one galactosyl side chain, were observed, but a fragment ion m/z 971, which was observed in the spectrum of peak A and formed through cleavage of a galactosyl–glucose side chain, was not detected. These phenomena suggest that all components are made up of seven glucosyl residues and one galactosyl residue, and they are doubly branched αCDs in which the galactosyl side chain is attached directly to the αCD ring of Glc-αCD.

^{13}C NMR spectroscopy.—Assignments of signals in the spectra of **Ia–If** and **IIa–IIe** were made by comparison with those in the spectra of Glc-αCD [3], 6-*O*-α-D-galactopyranosyl-αCD [1], and 2-*O*-α-D-galactopyranosyl-αCD (2-Gal-αCD) [4]. 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 [5]. The assignments of the C-6 signals were confirmed by the distortionless enhancement by polarization transfer (DEPT) method [6]. Gal-Glc-αCDs were clearly divided into two groups in their ^{13}C NMR spectra, that is, one group of **Ia–If** and another one of **IIa–IIe**. In the spectra of **Ia–If**, the large downfield shift of only one C-6 signal originated from the glucosyl side chain of Gal-Glc-αCDs was observed; therefore, it was suggested that a galactosyl side chain was attached to a carbon atom other than the C-6s of the CD ring glucoses. One of C-2 signals of the CD ring glucoses shifted downfield by 6–7 ppm from those of the other C-2s ($\delta \sim 72.0$), appearing at $\delta \sim 78.0$. The relative signal intensities of the CD ring C-6s (at $\delta \sim 61.3$), the glucosyl side chain C-6 (at $\delta \sim 61.5$), the galactosyl side chain C-6 (at $\delta \sim 61.7$), and the branch-point C-6 (at $\delta \sim 68.0$) were 5:1:1:1. Those of the CD ring C-4s (at δ 81.5–82.8), the galactosyl side chain C-4 (at $\delta \sim 69.3$), and the glucosyl side chain C-4 (at $\delta \sim 70.0$) were 6:1:1. Three types of C-1 signals appeared at a higher field than those of the C-1 signals of the αCD ring glucoses (δ 102.0–102.6): the α-(1 → 6)-linked C-1 signal of the glucose residue, the α-(1 → 2)-linked C-1 signal of the galactose residue, and the C-1 signal of the αCD ring glucose on which the galactosyl residue was attached at C-2. These were observed at $\delta \sim 99.8$, ~ 98.7, and ~ 99.4, respectively. From the above facts, it was confirmed that **Ia–If** were 2^n-*O*-α-D-galactopyranosyl-6^1-*O*-α-D-glucopyranosyl-αCDs.

On the other hand, in the spectra of **IIa–IIe**, the characteristic two kinds of signals, that is, the large downfield-shifted two branch-point C-6 signals (at δ 67.9–68.1) and the large upfield-shifted two C-1 signals (at δ 99.7–100.4), indicated that the galactosyl residue was substituted on C-6 of a ring glucose of Glc-αCD. Consequently, it was proved that **IIa–IIe** corresponded to 6^n-*O*-α-D-galactopyranosyl-6^1-*O*-α-D-glucopyranosyl-αCDs.

By ^{13}C NMR spectroscopy, the linkage mode of the galactosyl side chain of the

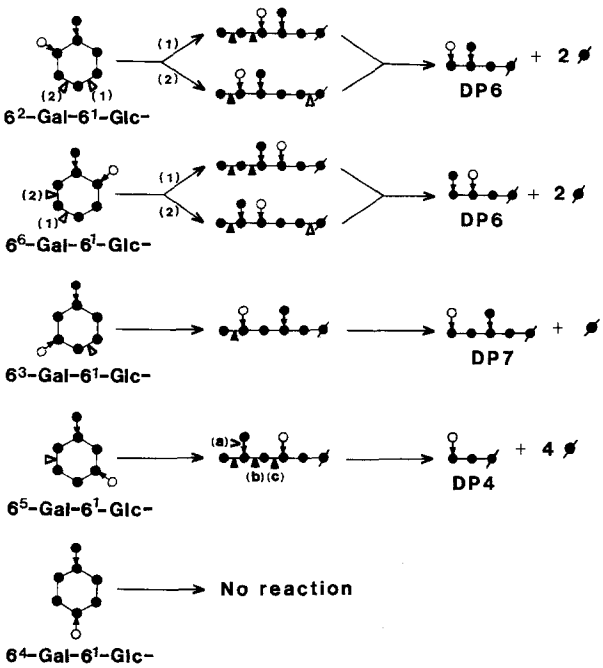

Fig. 2. Models of reactions on five positional isomers of 6^n-Gal-6^1-Glc-αCDs with CGTase and Glucoamylase. Symbols: ●, glucose; ◆, glucose with reducing end; ○, galactose; ●●, α-(1 → 4)-glucosidic linkage; ●●, α-(1 → 6)-glucosidic linkage; ●○, α-(1 → 6)-galactosidic linkage; ∧, attack point of glucoamylase; △, attack point of CGTase; ▲, attack point of CGTase and glucoamylase. At (b) and (c), the enzymes are able to act after attack of glucoamylase at (a).

Gal-Glc-αCDs could be determined, but the positional isomers were indistinguishable from each other.

Structural analysis.—6^n-Gal-6^1-Glc-αCDs (**II**): The structures of five positional isomers were elucidated by analyses of their digestion products with a combination of CGTase and glucoamylase. The degradation products of 6^n-Gal-6^1-Glc-αCDs (**IIa–IIe**) with these enzymes were analyzed by HPLC on an amino-bonded silica gel column, on which the retention times of saccharides increase with the increase of their molecular weights, and, moreover, their degree of polymerizations (dp's) were confirmed by FABMS. Kobayashi et al. [7] reported that 6^1,6^2- and 6^1,6^3-di-*O*-α-D-glucopyranosyl-αCDs were degradated with CGTase, whereas the 6^1,6^4-isomer was completely resistant to its action. On the basis of these data, we proposed the fragments that should be produced from 6^n-Gal-6^1-Glc-αCDs with CGTase and glucoamylase (see Fig. 2). Fig. 3 shows the chromatograms of the enzymatic degradation products of **IIa–IIe**, and peaks 1–4 correspond, respectively, to the oligosaccharides (**1–4**) produced. The dp of **1** was 7, and it was cleaved to galactose and an oligosaccharide of dp 6 with α-galactosidase. Therefore, it was revealed that **IIa** corresponded to 6^3-Gal-6^1-Glc-αCD. On the other hand, **2** and a large quantity of unchanged substance, namely 6^4-Gal-6^1-Glc-αCD, were detected in the degradation products of **IIb–c**. Compound **2** was 6^3-*O*-α-D-galactosyl-

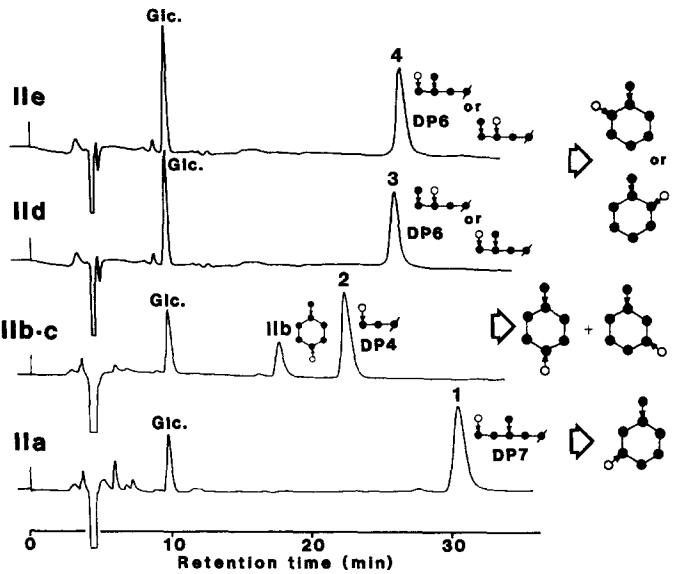

Fig. 3. Chromatograms of degradation products from **IIa–IIe** with CGTase and glucoamylase. Chromatographic conditions: column, YMC-Pack Polyamine-II (250×6 mm i.d.); eluent, 55:45 CH_3CN–H_2O; flow rate, 1 mL/min; temperature, 25 °C.

maltotriose, since it had dp 4 and was cleaved to galactose and maltotriose with α-galactosidase. Consequently, it was proved that **IIb–c** contained both of 6^4- and 6^5-Gal-6^1-Glc-αCDs. 6^4-Gal-6^1-Glc-αCD (**IIb**), which remained intact in decomposition of **IIb–c** with CGTase and glucoamylase, was purified by HPLC and confirmed by ^{13}C NMR spectroscopy. Next, **IId** and **IIe** were treated similarly, and **3** and **4** were detected as the degradation products, respectively. They had the same dp—dp 6;

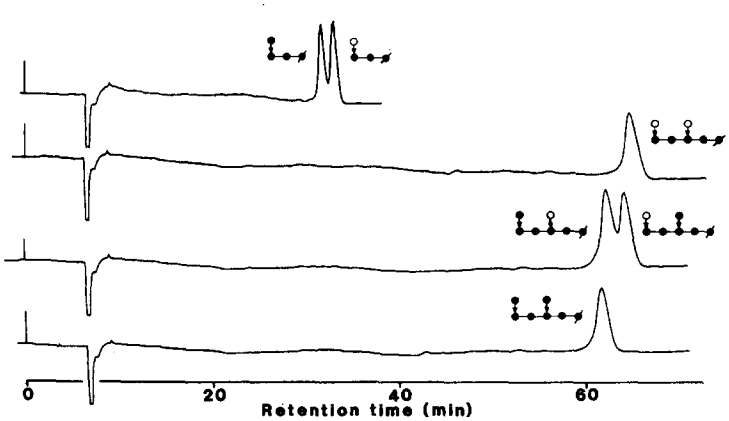

Fig. 4. Elution profiles of reference branched oligosaccharides. Chromatographic conditions: eluent, 60:40 CH_3CN–H_2O; flow rate, 0.7 mL/min; other conditions as in Fig. 3.

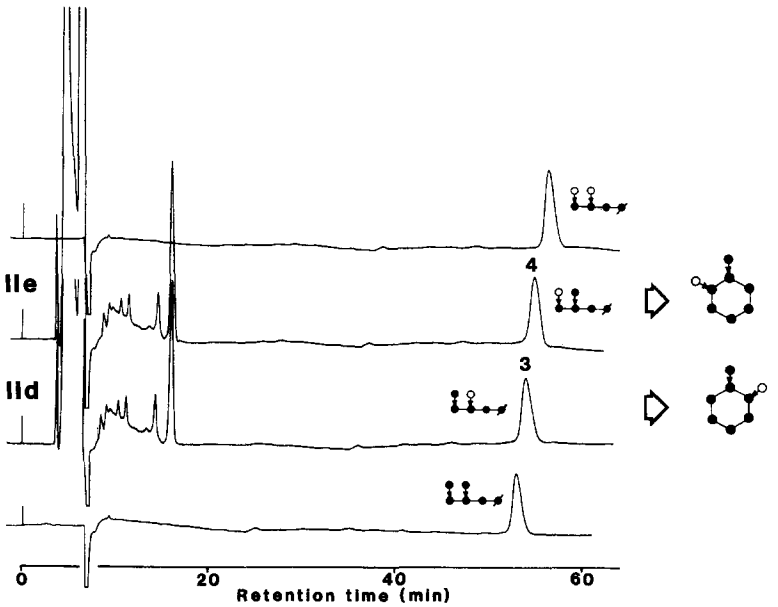

Fig. 5. Comparison of elution profiles of **3**, **4**, and two reference branched oligosaccharides. Chromatographic conditions as in Fig. 4.

therefore, it was concluded that they corresponded to either of 6^3-*O*-α-D-galactosyl-6^4-*O*-α-D-glucosyl- and 6^4-*O*-α-D-galactosyl-6^3-*O*-α-D-glucosylmaltotetraoses. α-Galactosidase or a large amount of glucoamylase was used in an attempt to distinguish between these branched oligosaccharides; however, the procedure was unsuccessful. Another method, their elution profile on HPLC, was employed whereby branched oligosaccharides of the same dp, having α-(1 → 6)-linked glucosyl and/or galactosyl side chains, were used for reference samples. These samples and their preparations are as follows. 6^3-*O*-α-D-Glucosylmaltotriose, 6^3,6^4-di-*O*-α-D-glucosylmaltotetraose, and 6^3,6^5-di-*O*-α-D-glucosylmaltopentaose were prepared by the digestion with CGTase of 6^1,6^4-, 6^1,6^2-, and 6^1,6^3-di-*O*-α-D-glucopyranosyl-βCDs, respectively. In a similar manner, 6^3-*O*-α-D-galactosylmaltotriose, 6^3,6^4-di-*O*-α-D-galactosylmaltotetraose, and 6^3,6^5-di-*O*-α-D-galactosylmaltopentaose were obtained from 6^1,6^4-, 6^1,6^2-, and 6^1,6^3-di-*O*-α-D-galactopyranosyl-βCDs, respectively [4]. 6^3-*O*-α-D-Galactosyl-6^5-*O*-α-D-glucosyl- and 6^5-*O*-α-D-galactosyl-6^3-*O*-α-D-glucosylmaltopentaoses were the digestion products of 6^5- and 6^3-*O*-α-D-galactosyl-6^1-*O*-α-D-glucosyl-αCDs with CGTase, respec-

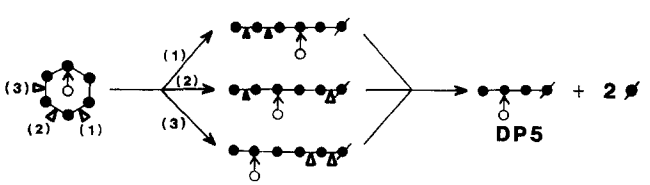

Fig. 6. Model of reaction on 2-Gal-αCD with CGTase and glucoamylase. Symbols: ●, α-(1 → 2)-galactosidic linkage; other symbols as in Fig. 2.

tively. These two heterogeneous branched oligosaccharides were distinguishable by the use of glucoamylase as mentioned above (see Fig. 2). It was confirmed that 6^3-*O*-α-D-glucosylmaltotriose eluted earlier than 6^3-*O*-α-D-galactosylmaltotriose, and of the 6^3,6^4-di-*O*-α-D-glucosyl- and 6^3,6^4-di-*O*-α-D-galactosylmaltotetraoses the former eluted earlier. Furthermore, 6^3,6^5-di-*O*-α-D-glucosylmaltopentaose eluted slightly earlier than 6^3-*O*-α-D-galactosyl-6^5-*O*-α-D-glucosylmaltopentaose, and 6^5-*O*-α-D-galactosyl-6^3-*O*-α-D-glucosyl- and 6^3,6^5-di-*O*-α-D-galactosylmaltopentaoses eluted in that order on a YMC-Pack Polyamine-II with an acetonitrile–water system as the eluent (Fig. 4). These phenomena suggest that the oligosaccharide having a glucosyl side chain on the non-reducing end was eluted earlier on this column than the isomer having a galactosyl side chain on the non-reducing end. Once again **3** and **4** were analyzed more precisely on a YMC-Pack Polyamine-II column, and it was confirmed that 6^3,6^4-di-*O*-α-D-glucosylmaltotetraose, **3**, **4**, and 6^3,6^4-di-*O*-α-D-galactosylmaltotetraose were eluted in that order (Fig. 5). Accordingly, it was probable that **3** and **4** were 6^3-*O*-α-D-galactosyl-6^4-*O*-α-D-glucosyl- and 6^4-*O*-α-D-galactosyl-6^3-*O*-α-D-glucosylmaltotetraoses, respectively, so that it was proposed that **IId** and **IIe** corresponded to 6^6- and 6^2-Gal-6^1-Glc-

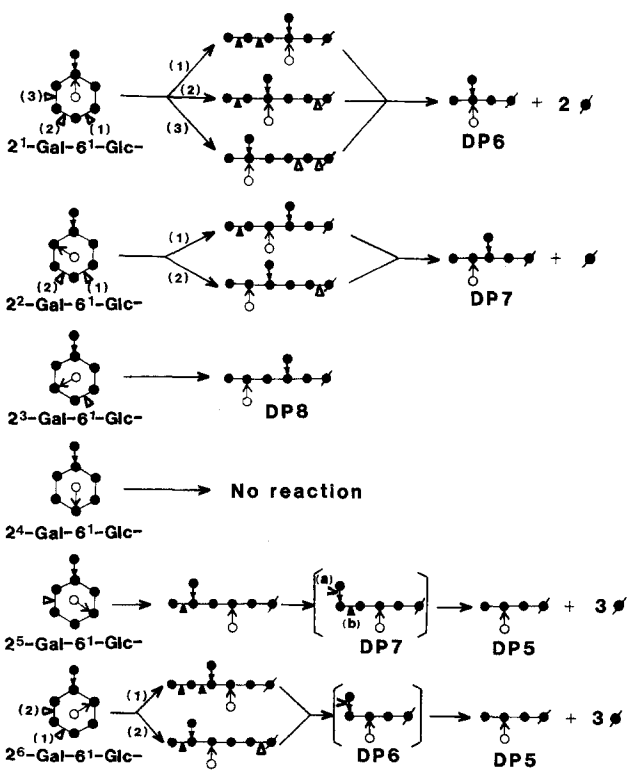

Fig. 7. Models of reactions on six positional isomers of 2^n-Gal-6^1-Glc-α-CDs with CGTase and glucoamylase. Symbols as in Fig. 6. At (b), the enzymes are able to act after attack of glucoamylase at (a).

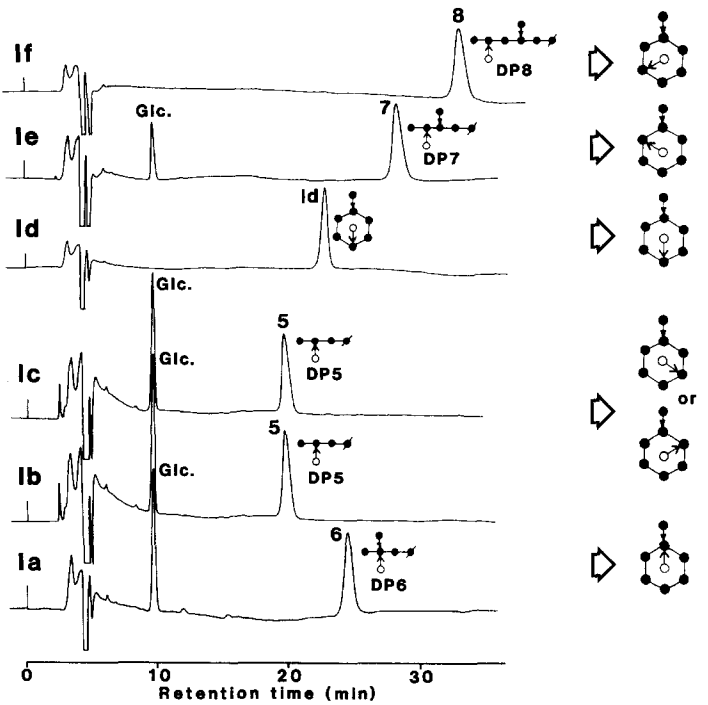

Fig. 8. Chromatograms of degradation products from **Ia–If** with CGTase and glucoamylase. Chromatographic conditions as in Fig. 3.

αCDs, respectively. Thus, it was proved that **IIa**, **IIb**, **IIc**, **IId**, and **IIe** were 6^3-, 6^4-, 6^5-, 6^6-, and 6^2-*O*-α-D-galactopyranosyl-6^1-*O*-α-D-glucopyranosyl-αCDs, respectively.

2^n-Gal-6^1-Glc-αCDs (**I**): Also the structures of six positional isomers of 2^n-Gal-6^1-Glc-αCDs (**Ia–If**) were elucidated in a similar manner as 6^n-Gal-6^1-Glc-αCDs. Prior to this, the degradation products of 2-Gal-αCD with CGTase and glucoamylase were analyzed, and an oligosaccharide (**5**) of dp 5 and glucose were detected. On the basis of this result, we proposed the model of degradation of 2-Gal-αCD with CGTase and glucoamylase (Fig. 6). It was supposed that CGTase and glucoamylase do not catalyze the hydrolysis of the non-reducing terminal of an α-(1 → 4)-glucosidic linkage adjacent to an α-(1 → 2)-galactosidic linkage in the molecules of heterogeneous branched oligosaccharides. By reference to this result and Fig. 2, the models of reaction of 2^n-Gal-6^1-Glc-αCDs with CGTase and glucoamylase are shown in Fig. 7. Fig. 8 shows the chromatograms of the hydrolysates of **Ia–If** with these enzymes, and the peaks 5–8, respectively, correspond to the oligosaccharides (**5–8**) produced. **Id** remained intact, while in the degradation products of **If** no glucose and only **8** (dp 8), which was formed by ring opening of 2^n-Gal-6^1-Glc-αCD, was detected. In the cases of **Ia** and **Ie**, glucose and **6** (dp 6), and glucose and **7** (dp 7) were observed, respectively. Accordingly, it turned out that **Ia**, **Id**, **Ie**, and **If** were 2^1-, 2^4-, 2^2-, and 2^3-Gal-6^1-Glc-αCDs, respectively. The same degradation products, that is, glucose and **5** (dp 5), were obtained

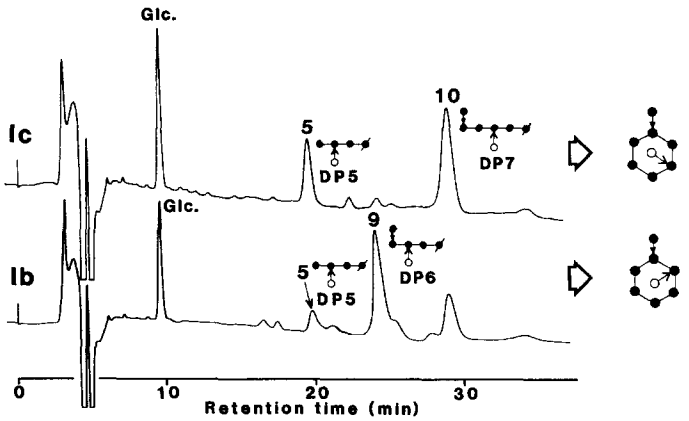

Fig. 9. Chromatograms of degradation products from **Ib** and **Ic** with CGTase and glucoamylase under rather mild conditions. Chromatographic conditions as in Fig. 3.

from **Ib** and **Ic**, so it was thought that they were either of 2^5- or 2^6-Gal-6^1-Glc-αCDs. Next, a rather mild condition of enzymatic degradation was applied to distinguish between **Ib** and **Ic**. It is known that glucoamylase hydrolyzes both α-(1 → 4)- and α-(1 → 6)-glucosidic linkages in starch, glycogen, and their hydrolysates; however, the hydrolysis rate of the α-(1 → 6)-linkage is slower than that of the α-(1 → 4)-linkage. When the quantity of glucoamylase used was reduced to 1/10, large amounts of **9** (dp 6) were detected as the intermediate product in decomposition of **Ib**, whereas **10** (dp 7) was the main product in the case of **Ic** (Fig. 9). From these facts, it was proved that **Ib** and **Ic** corresponded to 2^6- and 2^5-*O*-α-D-galactopyranosyl-6^1-*O*-α-D-glucopyranosyl-αCDs, respectively.

4. Conclusions

The six and five positional isomers of 2^n- and 6^n-Gal-6^1-Glc-αCDs, respectively, in the transgalactosylated products of Glc-αCD were isolated by HPLC using an ODS and a graphitized carbon columns. Particularly the latter column was useful for the fine separation of positional isomers, which were inseparable on the ODS column. The structures of all isomers were elucidated by enzymatic degradation with CGTase and glucoamylase. First of all, the rings of 2^n- and 6^n-Gal-6^1-Glc-αCDs were opened by CGTase, resulting in the formation of several heterogeneous branched oligosaccharides, though 2^4- and 6^4-Gal-6^1-Glc-αCDs remained intact. In 6^n-Gal-6^1-Glc-αCDs series, these doubly branched oligosaccharides were subsequently decomposed to fragments having an α-(1 → 6)-galactosidic linkage on the non-reducing end by CGTase and glucoamylase, except for 6^3-*O*-α-D-galactosyl-6^4-*O*-α-D-glucosylmaltotetraose. On the other hand, in 2^n-Gal-6^1-Glc-αCDs series, CGTase and glucoamylase did not attack on the non-reducing terminal of the α-(1 → 4)-glucosidic linkage adjacent to the branch by an α-(1 → 2)-galactosidic linkage in heterogeneous branched oligosaccharides; there-

fore, the fragments which had one α-(1 → 4)-glucosidic linkage on the non-reducing end were produced as the final products. Two series of positional isomers of heterogeneous branched αCDs and considerably unique branched oligosaccharides produced from the heterogeneous branched αCDs by enzymatic degradation should serve as novel standard samples in the fields of the enzymology and chemical synthesis of carbohydrates.

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