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Isolation and structural analyses of positional isomers of $6^1,6^n$-di-*O*-(*N*-acetyl-β-D-glucosaminyl)cyclomaltoheptaose ($n = 2$, 3, and 4) and 6-*O*-[6-*O*-(*N*-acetyl-β-D-glucosaminyl)-*N*-acetyl-β-D-glucosaminyl]-cyclomaltoheptaose

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Abstract

6-*O*-[6-*O*-(*N*-acetyl-β-D-glucosaminyl)-*N*-acetyl-β-D-glucosaminyl]cyclomaltoheptaose (βCD) and three positional isomers of $6^1,6^n$-di-*O*-(*N*-acetyl-β-D-glucosaminyl)cyclomaltoheptaose ($n = 2$, 3, and 4) in a mixture of products from βCD and *N*-acetylglucosamine by the reversed reaction of β-*N*-acetylhexosaminidase from jack bean were isolated and purified by HPLC. The structures of four isomers of di-*N*-acetylglucosaminyl-βCDs were determined by FABMS and NMR spectroscopy. The degree of polymerization of the branched oligosaccharides produced by enzymatic degradation with bacterial saccharifying α-amylase (BSA) was established by LC–MS methods.

Keywords: Di-*N*-acetyl-β-D-glucosaminyl-cyclomaltoheptaose (βCD); Positional isomer; Separation; Enzymatic degradation; Structural analysis; HPLC; LC–MS; NMR

1. Introduction

Cyclodextrins (CDs) have been extensively used to improve solubility, stability, and bioavailability of various water-insoluble compounds utilizing their complexation. Recently, to be useful in wider applications other than those of conventional CDs and homogeneous branched CDs such as glucosyl-CDs and maltosyl-CDs, a number of novel heterogeneous branched CDs that have an amino sugar as side chain were synthesized by the enzymatic reversed reaction of β-*N*-acetylhexosaminidase from jack bean. The main products were mono-branched CDs, 6-*O*-*N*-acetyl-β-D-glucosaminyl-CDs.[1] In this preparation, also the multi-substituted *N*-acetyl-β-D-glucosaminyl CDs as minor products should be produced.

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However, a detailed study on the multi-branched CDs has not yet been reported. *N*-Acetylglucosamine (GlcNAc) of the side chain is an important constituent of glycoconjugated substances as glycoproteins, glycolipids, and mucopolysaccharide in animal tissues. Further, GlcNAc on the terminal of sugar chains is recognized by special cells *in vivo*. On the other hand, we investigated inclusion behavior, solubility, and hemolytic activity of doubly branched CDs, 6^1,6^n-di-*O*-α-D-glucopyranosyl-βCDs ($n = 2$, 3, and 4) and found differences among the three positional isomers.[2] Moreover, it was also revealed that doubly branched βCDs had more advantages than mono-branched βCD, 6-*O*-α-D-glucopyranosyl-βCD, e.g., the hemolytic activity of doubly branched βCDs was lower than those of mono-branched βCD. Accordingly the novel multi-substituted *N*-acetylglucosaminyl CDs seem to be most interesting in the fields of pharmacy and biochemistry.

In the present study, four positional isomers of di-*N*-acetylglucosaminyl-βCDs [6^1,6^n-di-*O*-*N*-acetyl-β-D-glucosaminyl-βCDs (6^1,6^n-(GlcNAc)$_2$-βCDs); $n = 2$, 3, and 4 and 6-*O*-(6-*O*-*N*-acetyl-β-D-glucosaminyl)-*N*-acetyl-β-D-glucosaminyl-βCD (GlcNAc-(1→6)-GlcNAc-(1→6)-βCD)] in which two GlcNAc residues were substituted on βCD with β-*N*-acetylhexosaminidase [EC 3.2.1.52] from jack bean, were isolated and purified by HPLC. Further, the structures of these isomers were readily elucidated using FABMS, NMR spectroscopy, and an enzymatic degradation method.

2. Experimental

Materials.—A reversed reaction product of GlcNAc and βCD was prepared according to the previous paper.[1] Briefly, GlcNAc (12 g) and βCD (4.5 g) were dissolved in 50 mL of 20 mM acetate buffer (pH 5.0) and incubated with jack bean β-*N*-acetylhexosaminidase (500 U) at 50 °C for 3 days. Subsequently, the reaction mixture was heated at 100 °C for 10 min to stop the enzyme activity. Purified bacterial saccharifying α-amylase (BSA, EC 3.2.1.1) (400 U/mg) from *Bacillus subtilis*[3] and cyclomaltodextrin glucanotransferase (CGTase, EC 2.4.1.19) (2000 U/mL) from *B. circulans*[4] were prepared and purified by the method previously reported. All reagents were of analytical grade. HPLC-grade acetonitrile and methanol were used for chromatography. Water employed in the HPLC methods was purified using an Ultrapure water system, CPW-100 (ADVANTEC).

Isolation of di-N-acetylglucosaminyl-βCDs.—The fraction of di-*N*-acetylglucosaminyl-βCDs was separated from a mixture of mono-, di-, and tri-substituted *N*-acetylglucosaminyl-βCDs by HPLC on a CHEMCOBOND 5NH$_2$ column (250 × 20 mm i.d.) (Chemco) with 45:55 acetonitrile–water at a flow rate of 2.5 mL/min.

General methods.—HPLC was performed with a JASCO PU 980 pump, a Rheodyne 7125 injector, a RID-10A refractive index detector, and C-R6A chromatopac (SHIMADZU). HPLC analyses at constant temperature were conducted with an SSC 3510C column oven (Senshu Scientific Co.). The columns employed were a DAISOPAK SP-120-5-ODS-BP (250 × 20 mm i.d.) (DAISO), a Wakosil-II5C22 (250 × 4.6 mm i.d.) (Wako), and a Hypercarb 5μ (100 × 10 mm i.d.) (ThermoQuest). 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 Inc., San Jose, CA, USA) 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 capillary temperature was 250 °C. The pressure of the sheath gas was 70 psi, and the auxiliary gas was 15 unit. Total ion monitoring was done by scanning covered the range m/z 500–1500 with a scan rate of 3 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. NMR spectral data were recorded for 5–10% solutions in D_2O at 50 °C with a

JNM-ECP 400 spectrometer (JEOL). Chemical shifts were expressed in ppm downfield from the signal of Me_4Si referenced to external 1,4-dioxane (67.4 ppm). The other conditions for ^{13}C NMR, $^{1}H-^{1}H$ COSY and $^{1}H-^{13}C$ COSY measurements were the same as in the previous paper.[5]

Structural analyses by enzymatic degradation.—Each sample (0.2 mg) of di-*N*-acetylglucosaminyl-βCDs (**A**1, **A**2, **B**, and **C**) in 60 μL of 10 mM acetate buffer was individually incubated with BSA (0.8 mg, 320 U) or CGTase (20 μL, 40 U) at 40 °C for 48 h in order to digest completely. The enzyme was then inactivated by placing it in a boiling water bath for 10 min. The each hydrolysate with enzyme was analyzed by HPLC and LC–MS on a YMC-Pack Polyamine-II (150 × 4.6 mm i.d., YMC).

3. Results and discussion

Separation.—The reversed reaction product of βCD and GlcNAc with β-*N*-acetylhexosaminidase from jack bean was analyzed by HPLC using a CHEMCOBOND $5NH_2$ column with 45:55 acetonitrile–water (Fig. 1). The ratio of mono-*N*-acetylglucosaminyl-βCD

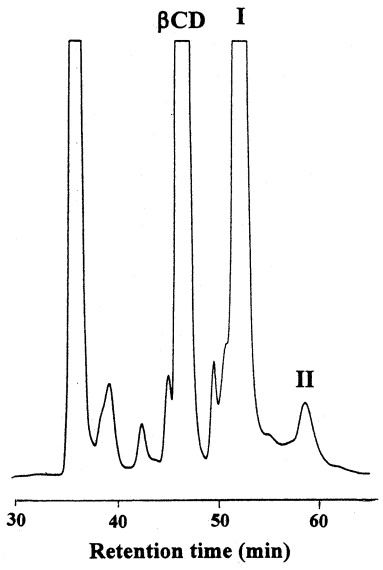

Fig. 1. Chromatogram of reversed reaction product of βCD and ClcNAc with β-*N*-acetylhexosaminidase from jack bean. (I) GlcNAc-βCD; (II) di-*N*-acetylglucosaminyl-βCDs. Chromatographic conditions: column, CHEMCOBOND $5NH_2$ (250 × 20 mm i.d.); eluent, 45:55 CH_3CN–water; flow rate, 2.5 mL/min; temperature, 30 °C.

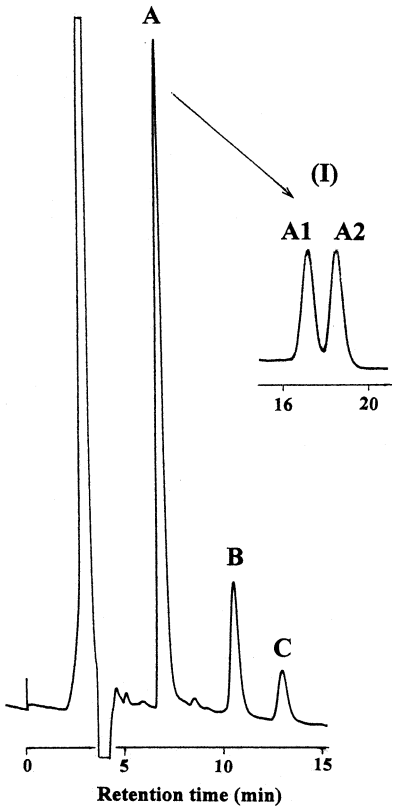

Fig. 2. Elution profile of di-*N*-acetylglucosaminyl-βCDs. Chromatographic conditions: column, Wakosil-II5C22 (250 × 4.6 mm i.d.); eluent, 8:92 CH_3OH–water; flow rate, 0.8 mL/min; temperature, 30 °C. Special conditions for separation of **A**1 and **A**2: (I) column, Hypercarb (100 × 10 mm i.d.); eluent, 12.5:87.5 CH_3CN–water; flow rate, 1.0 mL/min; temperature, 30 °C.

as the main product and di-*N*-acetylglucosaminyl-βCDs as the minor product was 19:1. Subsequently, the fraction of di-*N*-acetylglucosaminyl-βCDs corrected using a CHEMCOBOND $5NH_2$ column was separated to three components (**A**, **B**, and **C**) on a Wakosil-II5C22 column with 6:94 methanol–water, and the ratio of **A**:**B**:**C** was 72:19:9 (Fig. 2). First **A**, **B**, and **C** were isolated by a semi-preparative HPLC, using a DAISOPAK SP-120-5-ODS-BP column with 11:89 methanol–water at a flow rate 2.5 mL/min. Moreover, **A** was separated into **A**1 and **A**2 on a graphitized carbon column, a Hypercarb with 12.5:87.5 acetonitrile–water, and two components existed in equivalent (Fig. 2). Four components, **A**1, **A**2, **B**, and **C** of di-*N*-acetylglucosaminyl-βCDs, were isolated and purified by HPLC that employed two kinds of columns, an ODS and a graphitized carbon columns, and the ratio of **A**1:**A**2:**B**:**C** was found to be 36:36:19:9.

FABMS.—A molecular ion m/z 1539 was observed in each negative-ion FABMS spectrum of **A**1, **A**2, **B**, and **C**. Accordingly, it was confirmed that they were all comprised of βCD and two *N*-acetylglucosaminyl residues; that is, they were the positional isomers of di-*N*-acetylglucosaminyl-βCDs. Further, only one fragment ion m/z 1336 $[M-GlcNAc-H]^-$ formed through cleavage of either of two GlcNAc side chains was observed in the spectra of **A**1, **A**2, and **B**. On the other hand, in the spectrum of **C**, an additional fragment ion of m/z 1133 $[M-2\ GlcNAc-H]^-$ that formed through cleavage of one *N*-acetylglucosaminobiosyl side chain was detected. These data suggest that **A**1, **A**2, and **B** are the positional isomers of doubly branched *N*-acetylglucosaminyl-βCDs in which two GlcNAc side chains are attached directly to the βCD ring, while **C** is the *N*-acetylglucosaminobiosyl-βCD, that has only one side chain of an *N*-acetylglucosaminobiosyl group on the βCD ring.

^{13}C NMR spectroscopy

(1) N-Acetylglucosaminobiosyl-βCD. Fig. 3 shows the ^{13}C NMR spectrum of **C** together with that of 6-*O*-(*N*-acetyl-β-D-glucosaminyl)-βCD for reference. Their NMR spectra were assigned using ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY methods. 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] The assignments of the C-6 signals were confirmed by the distortionless enhancement by polarization transfer (DEPT) method.[7]

By FABMS analysis, it has been already estimated that **C** is *N*-acetylglucosaminobiosyl-βCD with another GlcNAc residue attached to side chain GlcNAc of 6-*O*-(*N*-acetyl-β-D-glucosaminyl)βCD. In the spectrum of **C**, four kinds of signals for the C-6 were observed: the C-6 signals of the βCD ring glucoses (G-6, δ 61.1–61.2), one C-6 signal (GN′-6, δ 61.8) of the terminal GlcNAc residue (GN′), and the two large downfield-shifted C-6 signals (G′-6 and GN-6, δ 67.9 and 69.2) of the branched point of βCD ring glucose (G′), and another GlcNAc residue (GN). The relative intensities of G-6, GN′-6, G′-6, and GN-6 were 6:1:1:1. Moreover, two β-(1→6)-linked C-1 signals (GN′-1 and GN-1) of the *N*-acetylglucosaminobiosyl side chains appeared at δ 102.3 and 101.6, respectively, in a more upfield position than G-1 and G′-1 (δ 102.6–102.7, 102.9). The relative intensities of G-1, G′-1, GN-1, and GN′-1 were 6:1:1:1. Therefore, it was determined that **C** was 6-*O*-[6-*O*-(*N*-acetyl-β-D-glucosaminyl)-*N*-acetyl-β-D-glucosaminyl]βCD [GlcNAc-(1→6)-GlcNAc-(1→6)-βCD].

(2) Doubly branched N-*acetylglucosaminyl-βCDs.* Fig. 4 shows the ^{13}C NMR spectra of **A**1, **A**2, and **B**. The signals for the C-6s of the five βCD ring glucoses (G-6, δ 61.1–61.2), C-6 signals of two GlcNAc side chains (GN-6, δ 61.7), and the two large downfield-shifted C-6 signals of the βCD ring glucoses (G′-6, δ 68.4–68.5) were observed. The relative signal intensities of three kinds of signals were 5:2:2. Similarly, three kinds of signals for C-1 were observed: the C-1 signals of the βCD ring glucoses (G-1, δ 102.6–102.7), the two downfield-shifted C-1 signals of the βCD ring glucoses (G′-1, δ 102.8–103.0), and the β-(1→6)-linked C-1 signals of two GlcNAc side chains (GN-1, δ 101.8–102.1) that appeared at a higher field than G-1 and G′-1. The relative signal intensities of G-1, G′-1, and GN-1 were 5:2:2. Therefore, it was confirmed that **A**1, **A**2, and **B** were the positional isomers of $6^1,6^n$-di-*O*-(*N*-acetyl-β-D-glucosaminyl)-βCD in which two GlcNAc side chains were attached to the βCD ring by a β-(1→6) linkage. Moreover, their spectra were compared in detail in order to estimate each structure of $6^1,6^2$-, $6^1,6^3$-, and $6^1,6^4$-isomers. In contrast to the cases of **A**1 and **A**2, each GN-1, -2, -3, -4, -5, and G′-1, -2, -3, -5 signal in that of **B** was clearly split into two peaks owing to the strong interaction between the two adjacent GlcNAc side chains substituted on the βCD ring. From this result, we propose that **B** corresponds to $6^1,6^2$-di-*O*-(*N*-acetyl-β-D-glucosaminyl)βCD.

Structural analysis.—It is well known that BSA and CGTase decompose α-(1→4)-glucosidic linkages except for the first and second glucosidic linkages toward the reducing end from the branching point [α-(1→6) glucosidic linkage] in starch and in branched dextrins,

These enzymes produce 6^3-*O*-α-D-glucosylmaltotriose (DP 4) as the smallest branched oligosaccharides. Using this feature we have already elucidated the structures of many positional isomers of homogeneous and heterogeneous multi-branched α, β, and γ CDs.[8–13] The highly rigid αCD ring, which has the smallest cavity size, is decomposed with CGTase, but not with BSA. On the other hand, the βCD and γCD rings are decomposed with BSA as well as CGTase due to their more flexible structure and larger cavity size than αCD.

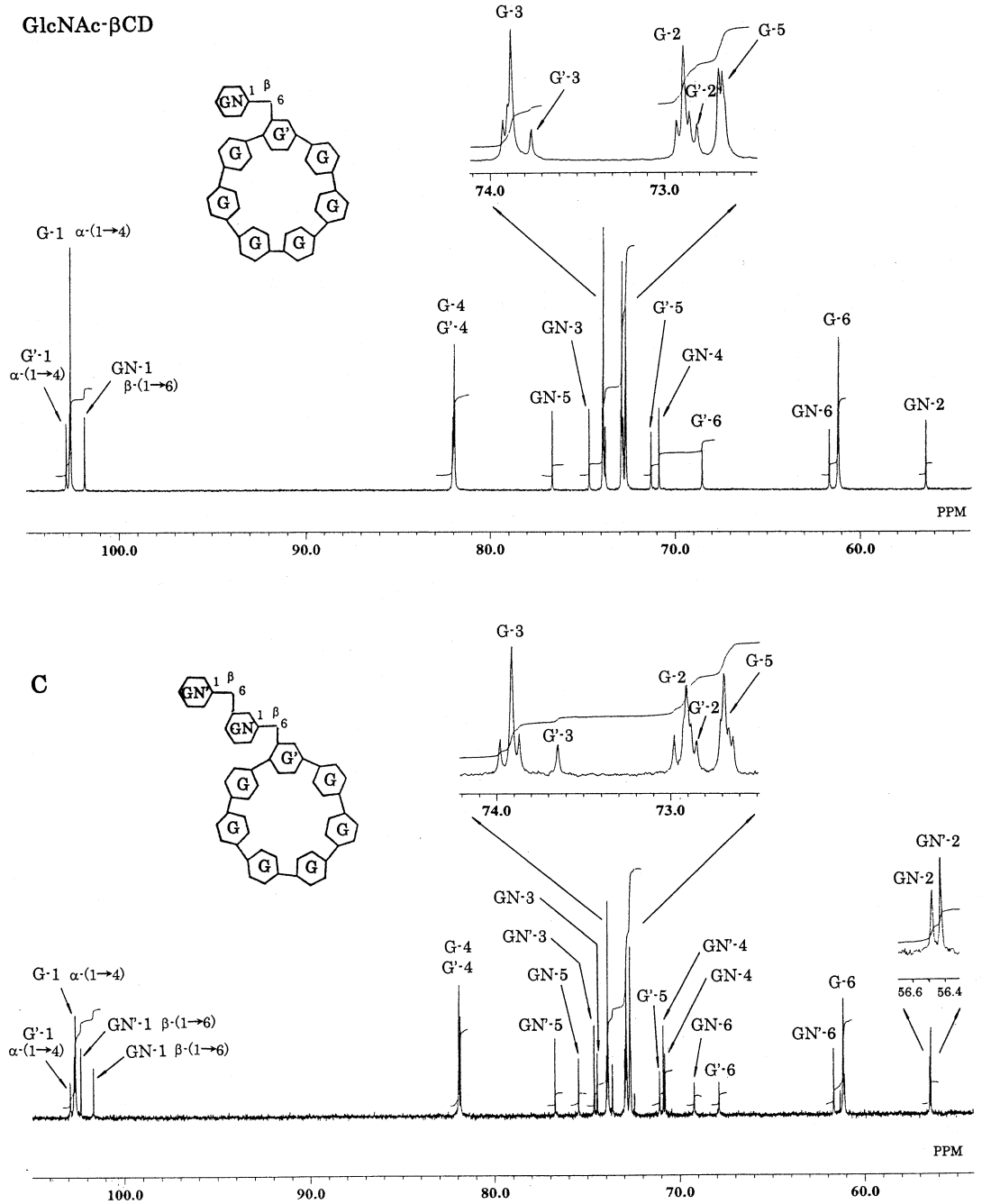

Fig. 3. ^{13}C NMR spectra of *N*-acetylglucosaminobiosyl-βCD (**C**) and GlcNAc-βCD in D_2O at 50 °C. G-1, -2, -3, -4, -5, and -6 are signals of C-1, -2, -3, -4, -5, and -6 atoms of the βCD ring D-glucopyranose units. G′ is the βCD ring D-glucopyranose unit on which the *N*-acetylglucosamine residue is β-(1→6)-linked. GN-1, -2, -3, -4, -5, and -6 are signals of C-1, 2, -3, -4, -5, and -6 atoms of the *N*-acetylglucosamine unit on which is β-(1→6)-linked directly to βCD. GN′ is the terminal *N*-acetylglucosamine unit of *N*-acetylglucosaminobiosyl side chain on βCD.

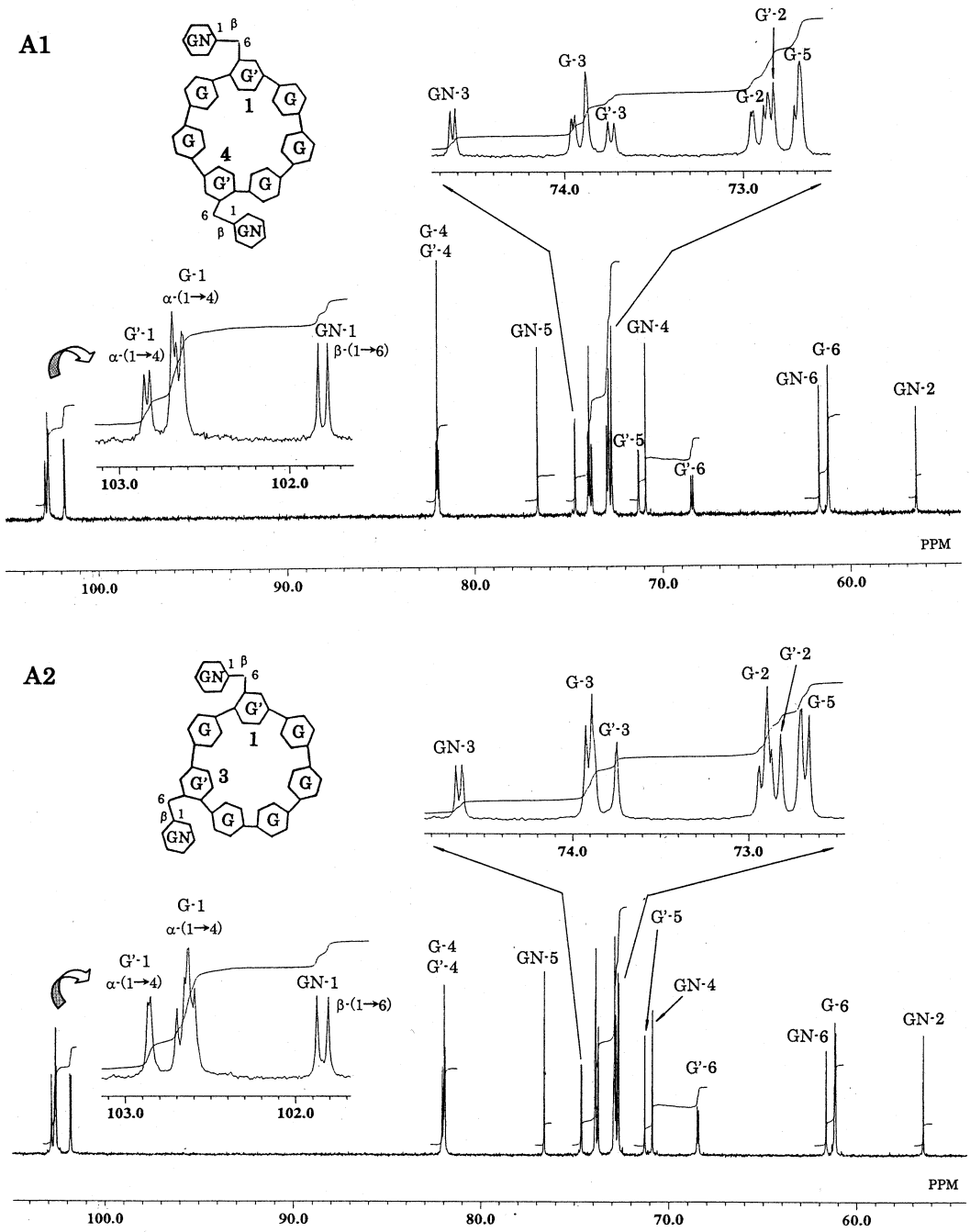

Fig. 4. ^{13}C NMR spectra of $6^1,6^n$-$(GlcNAc)_2$-βCDs (**A**1, **A**2, and **B**) in D_2O at 50 °C. The abbreviations are the same as in Fig. 3.

Initially we examined the enzymatic degradation of 6-*O*-(*N*-acetyl-β-D-glucosaminyl)-βCD (GlcNAc-βCD), because it was the fundamental compound of $6^1,6^n$-$(GlcNAc)_2$-βCDs and GlcNAc-(1→6)-GlcNAc-(1→6)-βCD. As expected, GlcNAc-βCD was finally decomposed to a DP 4 branched oligosaccharide and glucose with BSA. The same final degradation products were also detected in the digest with CGTase. On the basis of these results, the models of digestion of $6^1,6^n$-$(GlcNAc)_2$-βCDs and GlcNAc-(1→6)-GlcNAc-(1→6)-βCD with BSA are shown together with that of GlcNAc-βCD in Fig. 5. With BSA, the $6^1,6^2$-, $6^1,6^3$-, and $6^1,6^4$-isomers are decomposed to DP 6, 7, and 4 branched oligosaccharides, respectively, along with glucose. Furthermore, the DP 5 branched oligosaccharide and glu-

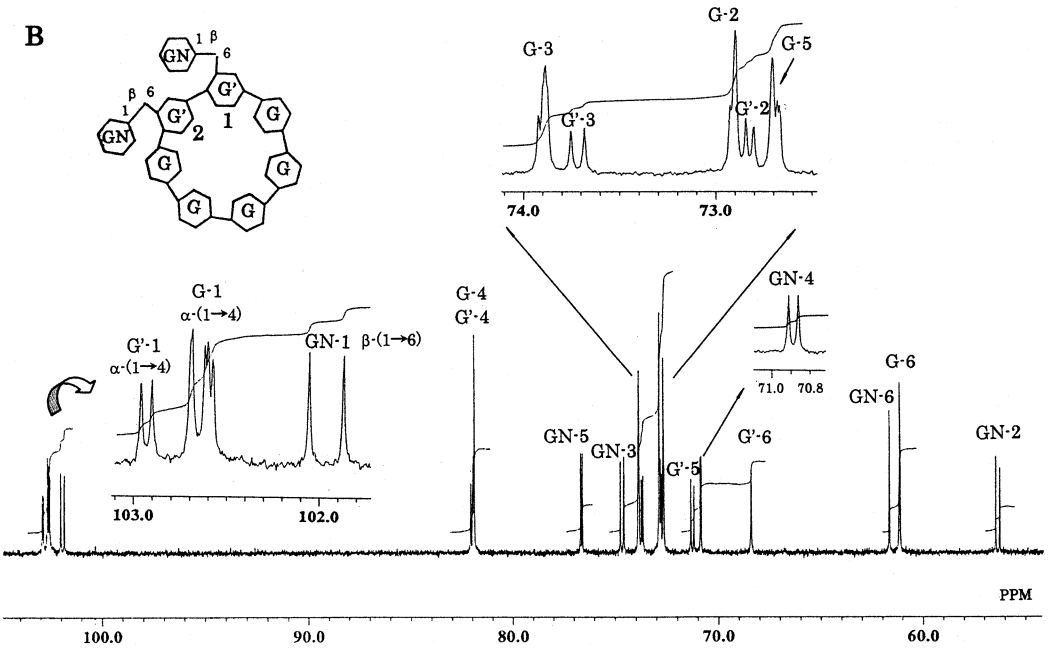

Fig. 4. (*Continued*)

cose are produced from GlcNAc-(1→6)-GlcNAc-(1→6)-βCD. Accordingly, three positional isomers of $6^1,6^n$-$(GlcNAc)_2$-βCDs and GlcNAc-(1→6)-GlcNAc-(1→6)-βCD could be easily elucidated by enzymatic degradation methods with BSA. They would give all the same results in the case of using CGTase as BSA. Fig. 6 shows the chromatograms by RI detection and the mass chromatograms of the enzymatic degradation products of **A**1, **A**2, **B**, and **C** with BSA. In the LC–MS analysis, the adduct ions with sodium ion $[M+Na]^+$ at m/z 730, 1257, 1095, and 933 for **1**–**4** that corresponded to the enzymatic digests of **A**1, **A**2, **B**, and **C**, respectively, were monitored in the positive-ion ESI mode. These results show that the products **1**–**4** are the branched oligosaccharides having DP 4, 7, 6, and 5, respectively. Thus, it was readily determined that **A**1, **A**2, and **B** were $6^1,6^4$-, $6^1,6^3$- and $6^1,6^2$-di-*O*-(*N*-acetyl-β-D-glucosaminyl)βCDs, respectively. On the other hand, the DP 5 (m/z 933) branched oligosaccharide was obtained from **C** (6-*O*-[6-*O*-(*N*-acetyl-β-D-glucosaminyl)-*N*-acetyl-β-D-glucosaminyl]βCD), as expected.

In addition, the structures determined with enzymatic degradation methods were consistent with those estimated previously with ^{13}C NMR spectroscopy.

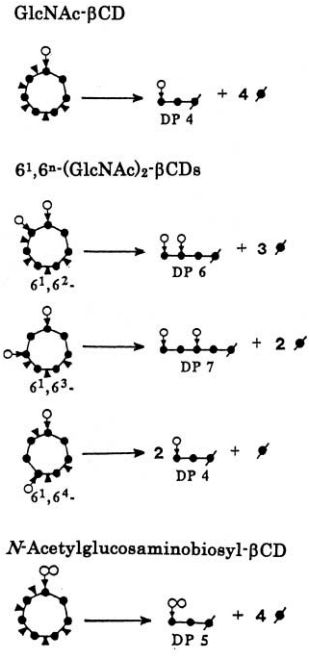

Fig. 5. Models of reaction on four positional isomers of di-*N*-acetyl-β-D-glucosaminyl-βCDs and GlcNAc-βCD with BSA. Symbols: ●, glucose; ; glucose with reducing end; ○, *N*-acetylglucosamine; ●-●, α-(1→4)-glucosidic linkage; ↓, β-(1→6) linkage between *N*-acetylglucosamine and glucose; ▲, attack point of BSA.

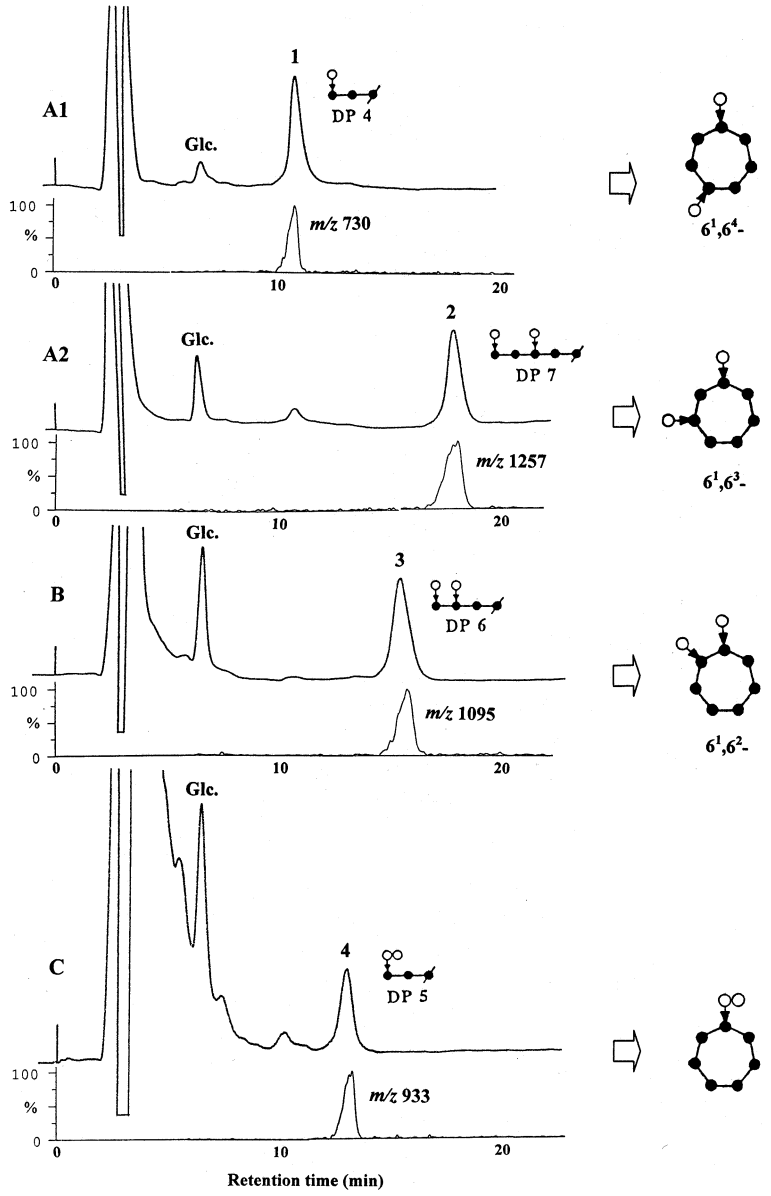

Fig. 6. Chromatograms of degradation products from **A**1, **A**2, **B**, and **C** with BSA and mass chromatograms of their sodium adducts. Chromatographic conditions: column, YMC-Pack Polyamine-II (150 × 4.6 mm i.d.); eluent, 57:43 CH_3CN–water; flow rate, 0.6 mL/min; temperature, 30 °C. Each upper chromatogram was detected by RI.

4. Conclusions

6-*O*-[6-*O*-(*N*-Acetyl-β-D-glucosaminyl)-*N*-acetyl-β-D-glucosaminyl]βCD and three positional isomers of $6^1,6^n$-di-*O*-(*N*-acetyl-β-D-glucosaminyl)βCD ($n = 2$, 3 and 4) were synthesized from βCD and *N*-acetylglucosamine by the reversed reaction of β-*N*-acetylhexosaminidase from jack bean. Four isomers of di-*N*-acetylglucosaminyl-βCDs were isolated and purified by HPLC successively using (A) an amino column and an acetonitrile–water system; (B) an ODS column and a methanol–water system; and (C) a graphitized carbon column and an acetonitrile–water system. In particular, the method C was required for separating $6^1,6^3$- and $6^1,6^4$-isomers among three isomers of $6^1,6^n$-$(GlcNAc)_2$-βCDs ($n = 2$, 3, and 4), because two isomers were difficult to separate on an ODS column using a methanol–water system. The respective structures of four isomers of di-*N*-acetylglucosaminyl-βCDs were determined by FABMS and NMR spectroscopy and by LC–MS determination of the degree of polymerization of the branched oligosac-

charides produced by enzymatic degradation with BSA or CGTase. Such a combination of instrumental analyses and enzymatic degradation should be a very powerful method for the structural determination of a series of novel multi-branched CDs.

It is likely that a series of these positional isomers of di-*N*-acetylglucosaminyl-βCDs will be very useful as standards in many related fields.

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