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Isolation, Purification, and Characterization of Cyclomaltotetradecaose (ι-Cyclodextrin), Cyclomaltopentadecaose (κ-Cyclodextrin), Cyclomaltohexadecaose (λ-Cyclodextrin), and Cyclomaltoheptadecaose (μ-Cyclodextrin)

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Although it has already been found that cyclodextrin glucanotransferase (CGTase) produces cyclodextrins (CDs) composed of six to thirteen glucopyranose units, the existence of CDs which have more than fourteen glucopyranose units has still not been confirmed. Cyclomaltotetradecaose (ι-CD), cyclomaltopentadecaose (κ-CD), cyclomaltohexadecaose (λ-CD) and cyclomaltoheptadecaose (μ-CD) are a new series of large-ring CDs composed of 14—17 α-(1→4)-linked D-glucopyranose units, respectively. ι-, κ-, λ- and μ-CD were purified from the commercially available CD powder produced by CGTase, by a combination of HPLC and column chromatography. The molecular weights of ι-, κ-, λ- and μ-CD were determined by FAB-MS, and their cyclic structures were identified by ^{1}H-NMR and ^{13}C-NMR. The ^{13}C-NMR chemical shifts of ι-, κ-, λ- and μ-CD were elucidated and compared with those of δ-, ε-, ζ-, η- and θ-CD, and their structures were predicted from the results.

Key words isolation; purification; ι-cyclodextrin; κ-cyclodextrin; λ-cyclodextrin; μ-cyclodextrin

Cyclodextrins (CDs) are a series of cyclomalto-oligosaccharides and conventional α-, β- and γ-CD are commercially produced using cyclodextrin glucanotransferase (CGTase). These conventional CDs and their derivatives have been well studied and used by various industries.[1-4] In pharmaceutical preparations, they are useful in improving drug stability, solubility, dissolution rate, bioavailability, and reduction of the side effects of drugs, and their application and safety profiles have been reviewed.[5-9] On the other hand, there have been few papers concerning large-ring CDs (LR-CDs) composed of more than nine α-(1→4)-linked D-glucopyranose units, except for a paper by French *et al.* in 1965 which was the first definitive evidence for the existence of such molecules of which numbers 9, 10, 11, 12 and 13 are named δ-, ε-, ζ-, η-, and θ-CD, respectively.[10] Recently, we also isolated and purified the above five kinds of LR-CDs from a commercially available CD powder produced by CGTase and reconfirmed their existence by modern analytical techniques, showing that δ-CD had lower aqueous solubility and much weaker hemolytic activity than α-CD or γ-CD, and conferring complex-forming ability for some drugs.[11-13] However, it was not known whether LR-CDs composed of more than fourteen glucopyranose units were produced by CGTase, although there have been papers of glucoamylase-resistant molecules in amyloses synthesized with bacterial α-amylase and potato D-enzyme.[14,15] In this report, the isolation, purification, and characterization of cyclomaltotetradecaose (ι-CD), cyclomaltopentadecaose (κ-CD), cyclomaltohexadecaose (λ-CD) and cyclomaltoheptadecaose (μ-CD) from a CD mixture produced by CGTase are described. The ^{13}C-NMR chemical shifts of four new kinds of LR-CDs (ι-, κ-, λ-, μ-CD) were also elucidated and compared with those of other LR-CDs (δ-, ε-, ζ-, η-, θ-CD), and their structures were predicted from these results.

Experimental

Materials CD powder (DEXY PEARL K-50), β-amylase [α-(1→4)-glucan maltohydrolase], and glucoamylase [α-(1→4)-glucan glucohydrolase] were purchased from Ensuiko Sugar Refining Co. (Yokohama, Japan), Tokyo Kasei Kogyo Co. (Tokyo, Japan), and Seikagaku Kogyo Co. (Tokyo), respectively. Novo Nordisk Bioindustry Co. (Chiba, Japan) donated pullulanase [α-(1→6)-glucosidase, Promozym 200L™]. All other chemicals were from reliable commercial sources and were used without further purification. Milli-Q water (Millipore Co., Milford, MA, U.S.A.) was used as purified water in all preparations and purifications.

Apparatus and Columns for Preparative and Analytical Methods HPLC was performed on a preparative octadecyl bonded silica (ODS) column with a SSC Flow System 3100J pump (Senshu Kagaku, Tokyo), with a DEGASYS DA-1200 degasser (Uniflows, Tokyo), and an ERC-7530 refractive index (RI) monitor (Erma Optical Works, Tokyo). HPLC on a preparative amino bonded (NH_2) column and HPLC analyses of CDs were performed using a PU-986 intelligent pump (Jasco, Tokyo), with a DG-980-50 3-line degasser (Jasco), and RI-930 intelligent RI detector (Jasco) with an 807-IT integrator (Jasco). In HPLC experiments, the column temperature was held constant with a water bath and a column oven. For preparative chromatography, the columns used for HPLC were a Senshu Pak ODS-5251-SS (250 mm × 20 i.d., Senshu Kagaku) and an Asahipak NH2P-50 (250 mm × 10 i.d. and 250 mm × 4.6 i.d., Showa Denko, Tokyo). The columns used for analytical HPLC were Hibar LiChroCART LiChrospher 100 RP-18 (e) (250 mm × 4 i.d., Cica-Merck, Darmstadt, Germany) and Asahipak NH2P-50 (250 mm × 4.6 i.d., Showa Denko).

Preparation of LR-CD Mixture The procedure for preparation of LR-CD mixture was described in detail previously.[11] Briefly, using the enzyme-catalyzed reaction with β-amylase, pullulanase and yeast in acetate buffer (pH 5.2) and complexation with bromobenzene, tetrachloroethane and ethanol, α-, β-, γ-CD, their derivatives and other oligosaccharides were removed from commercially available CD powder (DEXY PEARL K-50). The supernatant seemed to contain many kinds of LR-CDs and was subjected to deionization, decolorization and hydrolysis using glucoamylase; then a LR-CD mixture was precipitated by acetone.

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Isolation and Purification of ι-, κ-, λ- and μ-CD A LR-CD mixture was redissolved in water and was roughly separated into 4 fractions by HPLC on a preparative ODS column with methanol–water (6:100) at a flow rate of 6.0 ml/min as described previously.[11] Fraction HO-2 was further fractionated into 4 fractions by HPLC on a semi-preparative Asahipak NH2P-50 with acetonitrile–water (55:45) at a flow rate of 2.0 ml/min because this fraction was rich in LR-CDs.[12,13] Finally, ι-CD (NH-3-1) and κ-CD (NH-3-2) were purified by HPLC on the semi-preparative Asahipak NH2P-50 from fr. NH-3, and λ-CD (NH-4-1) and μ-CD (NH-4-2) were purified by HPLC on the analytical Asahipak NH2P-50 from fr. NH-4.

Purity of ι-, κ-, λ- and μ-CD by HPLC Their purities were checked by HPLC on a reverse phase column (LiChrospher 100 RP-18 (e)) and a normal phase column (Asahipak NH2P-50). HPLC was performed under the following conditions: (1) column, LiChrospher 100 RP-18 (e); eluent, methanol–water (5:95); flow rate, 0.8 ml/min; column temperature, 25 °C, (2) column, Asahipak NH2P-50; eluent, acetonitrile–water (55:45); flow rate, 0.7 ml/min; column temperature, 20 °C. The HPLC apparatus was identical to that described in the section on apparatus and columns for preparative and analytical methods.

Characterization of ι-, κ-, λ- and μ-CD by Mass Spectra FAB-MS spectra were measured in a positive ion mode by an SX-102A mass spectrometer (JEOL, Tokyo) using Magic Bullet as a matrix. The acceleration voltages were 10 kV for ι-CD and κ-CD, and 7 kV for λ-CD and μ-CD.

Characterization of ι-, κ-, λ- and μ-CD by NMR Spectra ^{1}H-NMR, ^{13}C-NMR and two-dimensional ^{1}H–^{13}C correlation (H, C COSY) NMR spectra were taken on a JNM-LA500 spectrometer (500 MHz ^{1}H, JEOL) at 50 °C. The samples were dissolved in 99.8% deuterium oxide. Chemical shifts were reported in δ-units (ppm) downfield from the signal of external tetramethylsilane [$(CH_3)_4Si$].

Results and Discussion

Isolation and Purification of ι-, κ-, λ- and μ-CD The LR-CD mixture was prepared from CD powder and separated into four fractions on the preparative ODS column to obtain the LR-CD-rich fraction in a manner similar to that described previously.[11] In these fractions, fr. HO-2 was collected and analyzed by HPLC on an Asahipak NH2P-50, since it has already been shown that the LR-CDs from ε-CD to θ-CD were included in fr. HO-2.[12,13] Figure 1 shows the chromatogram of fr. HO-2 by HPLC on an analytical Asahipak NH2P-50. This suggests that some components with larger retention times than θ-CD in fr. HO-2 would be larger CD, because generally the elution sequence with an aminopropyl-bonded silica column and acetonitrile-water system follows the order of number of glucopyranose units, and the Asahipak NH2P-50 had almost same the elution sequence.[16] In the next purification step, fr. HO-2 was further separated into four fractions by HPLC on semi-preparative Asahipak NH2P-50, as shown in Fig. 1. Finally, ι-CD (NH-3-1) and κ-CD (NH-3-2) were purified by HPLC on the semi-preparative Asahipak NH2P-50 from fr. NH-3, and λ-CD (NH-4-1) and μ-CD (NH-4-2) were purified by HPLC on the analytical Asahipak NH2P-50 from fr. NH-4. Other peaks in fr. NH-4 (NH-4-3, 4, 5) might also have been larger LR-CD considering the elution sequence on the analytical Asahipak NH2P-50 and FAB-MS measurement. The characterization of these compounds is currently under way and results will be published in the near future.

Purity of ι-, κ-, λ- and μ-CD Each LR-CD was analyzed by HPLC on a reverse phase column (LiChrospher 100 RP-18 (e)) and normal phase column (Asahipak NH2P-50). Figure 2 shows the chromatograms of ι-CD as a representative example, and this LR-CD had >98% purity; κ-, λ- and μ-CD had similar purity. We obtained about 200 mg of ι-CD, about 100 mg of κ-CD and about 10 mg of λ-CD and μ-CD. The yields of ι-CD and κ-CD based on about 10.88 kg of CD powder were 1.8×10^{-3} % and 9.2×10^{-4} %, respectively, and those of λ-CD and μ-CD based on about 2.56 kg of CD powder were 3.9 ×

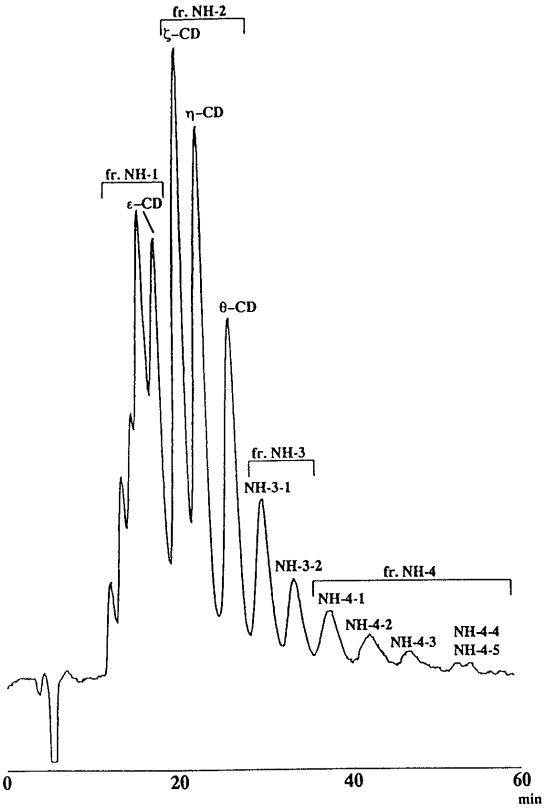

Fig. 1. Chromatogram of Fr. HO-2 on an Analytical Asahipak NH2P-50

Conditions: column, Asahipak NH2P-50; eluent, CH_3CN–H_2O (55:45); flow rate, 0.7 ml/min; column temperature, 20 °C.

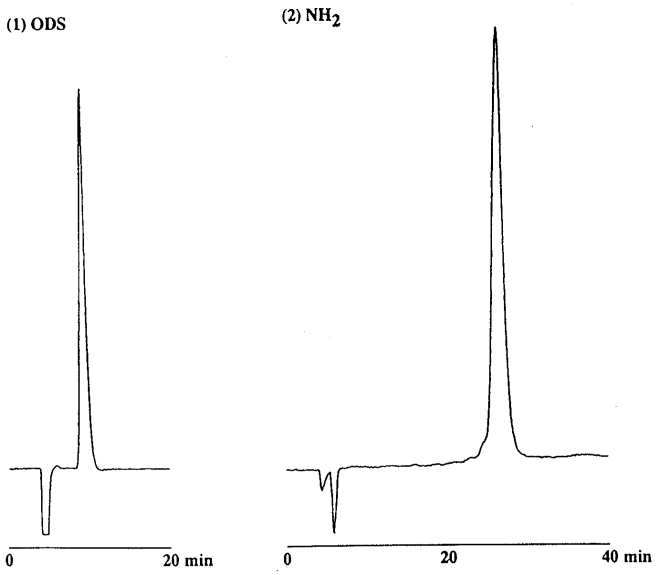

Fig. 2. Chromatograms of ι-CD on an ODS and an NH_2 Column

Conditions: (1) Column, LiChrospher 100 RP-18 (e); eluent, CH_3OH–H_2O (5:95); flow rate, 0.8 ml/min; column temperature, 25 °C. (2) Column, Asahipak NH2P-50; eluent, CH_3CN–H_2O (55:45); flow rate, 0.7 ml/min; column temperature, 20 °C.

10^{-4}%.

Characterization of ι-, κ-, λ- and μ-CD by HPLC, FAB-MS and NMR To obtain the elution sequence of new LR-CDs on the Asahipak NH2P-50, the four freshly prepared LR-CDs were subjected to HPLC under the same conditions. Figure 3 shows the chromatogram of four kinds of LR-CDs on the Asahipak NH2P-50. The elution sequence followed the number of glucopyranose units, which indicated that the number of these units increased in the following order: ι-CD < κ-CD < λ-CD < μ-CD.

Figure 4 shows the FAB-MS spectrum of ι-CD as a representative example. Each FAB-MS spectrum gave the following: *m/z* peak 2269.6 $[M+H]^+$ and 2290.2 $[M+Na]^+$ from ι-CD, *m/z* peak 2432.4 $[M+H]^+$ and 2453.9 $[M+Na]^+$ from κ-CD, *m/z* peak 2595.0 $[M+H]^+$ and 2618.0 $[M+Na]^+$ from λ-CD and *m/z* peak 2758.0 $[M+H]^+$ and 2780.1 $[M+Na]^+$ from μ-CD. These findings were in agreement with the calculated molecular weight of ι-CD (Calcd for $(C_6H_{10}O_5)_{14}$: 2268.740), κ-CD (Calcd for $(C_6H_{10}O_5)_{15}$: 2430.792), λ-CD (Calcd for $(C_6H_{10}O_5)_{16}$: 2592.845) and μ-CD (Calcd for $(C_6H_{10}O_5)_{17}$: 2754.898), and indicated that the four obtained LR-CDs were composed of 14, 15, 16 and 17 glucopyranose units, respectively.

The ^{1}H-NMR, ^{13}C-NMR spectrum and two-dimensional ^{1}H–^{13}C correlation (H, C COSY) NMR spectra of the four obtained LR-CDs were measured with a

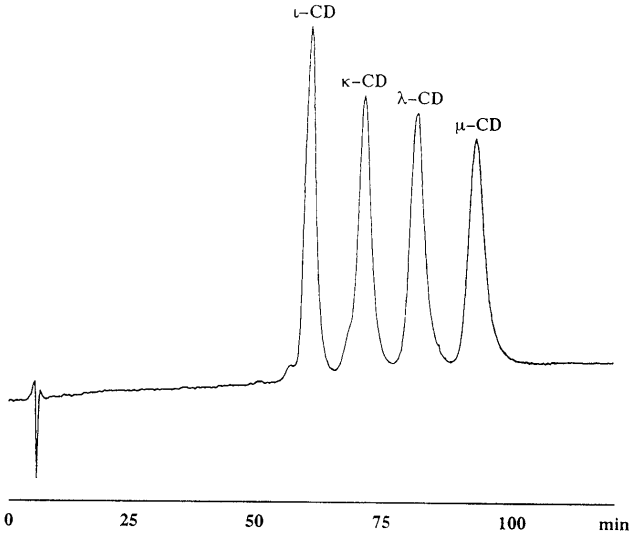

Fig. 3. Elution Profile of ι-, κ-, λ- and μ-CD on an Asahipak NH2P-50

Conditions: column, Asahipak NH2P-50; eluent, CH_3CN–H_2O (60:40); flow rate, 0.7 ml/min; column temperature, 20 °C.

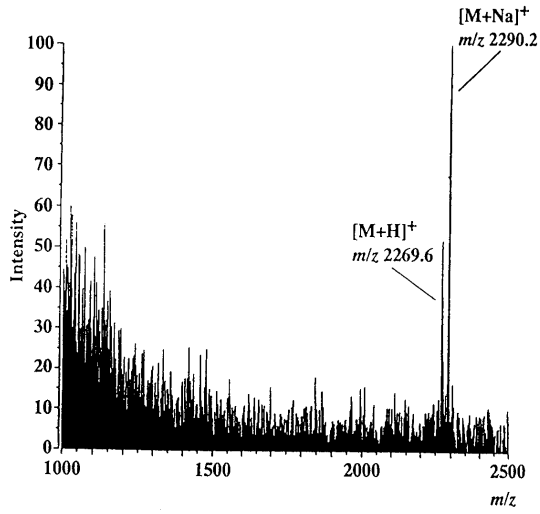

Fig. 4. FAB-MS Spectrum of ι-CD

Matrix, Magic Bullet; acceleration voltage, 10 kV.

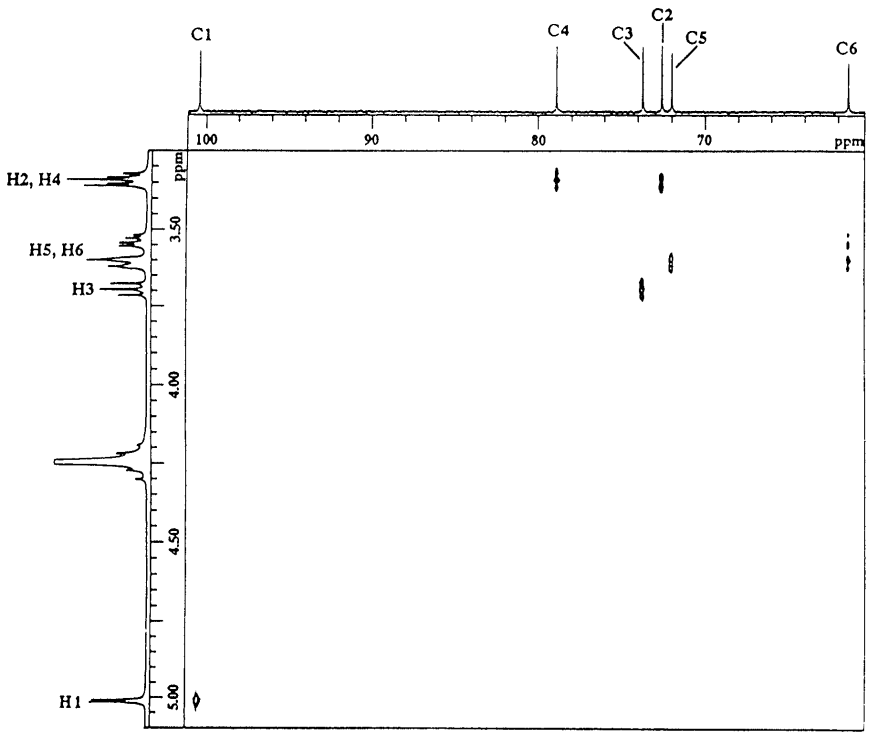

Fig. 5. ^{1}H–^{13}C COSY NMR Spectrum of ι-CD

Solvent, deuterium oxide; temperature, 50 °C.

Table 1. ^{13}C-NMR Chemical Shifts of LR-CDs

Carbon	δ value (ppm)								
	δ-CD	ε-CD	ζ-CD	η-CD	θ-CD	ι-CD	κ-CD	λ-CD	μ-CD
1	100.96	99.74	99.83	100.17	100.34	100.43	100.29	100.08	100.07
2	73.08	72.65	72.56	72.53	72.51	72.56	72.59	72.54	72.48
3	73.74	73.68	73.66	73.67	73.64	73.69	73.76	73.82	73.88
4	79.26	78.03	78.31	78.87	78.97	78.89	78.51	78.09	78.00
5	72.34	71.78	71.74	71.82	71.90	71.97	72.00	71.97	71.96
6	61.29	61.49	61.53	61.54	61.43	61.39	61.40	61.42	61.44

ppm downfield from external tetramethylsilane at 50 °C in D_2O solution.

JNM-LA500 spectrometer at 50 °C in deuterium oxide. Figure 5 shows the ^{1}H–^{13}C correlation (H, C COSY) NMR spectrum of ι-CD as a representative example. Their assignments could be made from the two-dimensional ^{1}H–^{13}C correlation (H, C COSY) NMR spectra, and a free –CH_2OH group could be discerned. The spectra of ι-, κ-, λ- and μ-CD each showed six clear and distinct single peaks attributable to equivalent glucopyranose units in the ^{13}C-NMR spectrum. Chemical shifts of each carbon obtained with ι-, κ-, λ- and μ-CD were similar to those that had been attributed to the cyclic structures of other LR-CDs except for δ-CD (see Table 1). Figure 6 shows the values of the chemical shifts at each carbon in twelve kinds of CDs. The chemical shifts of C1 and C4 were shifted upfield, although those of the other four carbons showed little shift. Further, the values of chemical shift of C1 and C4 were divided into three groups: conventional CDs, δ-CD, and other LR-CDs. C1 and C4 are used for binding to two glucopyranose units and these variations might have been caused by a difference in the state of α-(1→4) glucosidic linkage. All CDs reported to date may have the same fundamental cyclic structure of α-(1→4) glucosidic linkage, but conventional CDs, δ-CD, and other LR-CDs have some differences in fine structure. NMR measurements suggested a distorted cyclic structure of LR-CDs, and some of the linkages at the 1- and 4-positions were in a nonidentical state. This speculation was supported by the crystal and molecular structure of δ-CD $13.75H_2O$,[17] ε-CD $19H_2O$[18] and ι-CD; that is, structural features were a wreath-shaped truncated cone for conventional CDs and a saddle-like structure for ε-CD and ι-CD. The detailed molecular structure of ι-CD has been determined.[19]

In conclusion, from commercially available CD powder produced by CGTase we isolated and for the first time purified ι-, κ-, λ- and μ-CD, and their identities were confirmed by modern analytical techniques including HPLC, FAB-MS, and one- and two-dimensional NMR. We earlier confirmed the presence of δ-, ε-, ζ-, η-, and θ-CD from the same source. These results indicated that CGTase could produce larger CDs than θ-CD along with conventional CDs and other LR-CDs as well as bacterial α-amylase and potato D-enzyme. The ^{13}C-NMR data further suggested that structures of the four new LR-CDs were similar to those of ε-, ζ-, η- and θ-CD.

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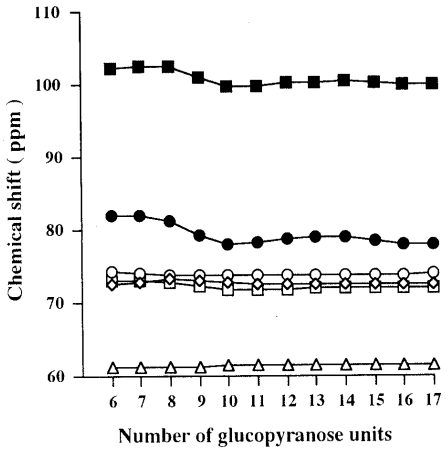

Fig. 6. Variation in ^{13}C Chemical Shifts of CDs with Number of Glucopyranose Units

■, C1; ◇, C2; ○, C3; ●, C4; □, C5; △, C6.

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