Isolation, Purification and Characterization of Cyclomaltooctadecaose (ν-Cyclodextrin), Cyclomaltononadecaose (ξ-Cyclodextrin), Cyclomaltoeicosaose (o-Cyclodextrin) and Cyclomaltoheneicosaose (π-Cyclodextrin)[1)]

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Under standard conditions, *i.e.* use of natural starches as substrates and long reaction times, it has been confirmed that cyclodextrin glucanotransferase (CGTase) produces not only conventional α-, β- and γ-cyclodextrins (CDs) but also large-ring CDs (LR-CDs) composed of 9 to 17 glucopyranose units as minor products. Recently, it was reported that CGTase produced cyclic α-1,4-glucans with degrees of polymerization ranging from 9 to more than 60 units under different reaction conditions such as the use of synthetic amyloses as substrates and short reaction times. However, the maximum size of LR-CDs which can be produced by CGTase under standard conditions, has not yet been determined. Therefore, in the present study we searched for LR-CDs composed of more than 18 glucopyranose units in commercially available CD powder produced by CGTase from potato starch. We found 4 new kinds of LR-CD, cyclomaltooctadecaose (ν-CD), cyclomaltononadecaose (ξ-CD), cyclomaltoeicosaose (o-CD) and cyclomaltoheneicosaose (π-CD), and these were purified by a combination of HPLC and column chromatography. Their molecular weights were determined by FAB-MS, and their α-(1→4)-linked cyclic structures were identified by ^{1}H-NMR and ^{13}C-NMR. The ^{13}C-NMR chemical shifts of these new LR-CDs were elucidated and compared with 9 kinds of previously reported LR-CDs. Their structures were predicted to be similar to those of cyclomaltodecaose (ε-CD) and cyclomaltotetradecaose (ι-CD). These results indicate that CGTase can produce LR-CDs consisting of up to about 20 glucopyranose units under standard reaction conditions.

Key words isolation; purification; ν-cyclodextrin; ξ-cyclodextrin; o-cyclodextrin; π-cyclodextrin

Cyclodextrins (CDs) are cyclomalto-oligosaccharides produced by cyclodextrin glucanotransferase (CGTase), and it has been shown that conventional α-, β- and γ-CD are the main products of this enzyme. These conventional CDs and their derivatives have been widely studied and used.[2—4)] Recently, we isolated and purified 9 kinds of large-ring CDs (LR-CDs), composed of 9 to 17 glucopyranose units (GUs) and named δ-, ε-, ζ-, η-, θ-, ι-, κ- λ- and μ-CD, respectively, from commercially available CD powder produced by treating potato starch with CGTase.[5—8)] Furthermore, we also reported the physicochemical properties and complex-forming ability of δ-CD[5)] and X-ray crystal structures of ε- and ι-CD.[9,10)] On the other hand, it has been reported that bacterial α-amylase and potato D-enzyme synthesized glucoamylase-resistant molecules from amylose[11,12)] and CGTase can produce cyclic α-1,4-glucans with degrees of polymerization ranging from 9 to more than 60 units together with conventional α-, β- and γ-CD under different reaction conditions, such as the use of synthetic amylose as a substrate and short reaction times.[13)] However, the maximum size of LR-CDs which can be produced by CGTase under standard reaction conditions, such as the use of natural starches as substrates and long reaction times, is unknown. To resolve this issue, we analyzed commercially available CD powder for LR-CDs composed of more than 18 GUs and found four new kinds of LR-CD, cyclomaltooctadecaose (ν-CD), cyclomaltononadecaose (ξ-CD), cyclomaltoeicosaose (o-CD) and cyclomaltoheneicosaose (π-CD). Their molecular weights were determined by FAB-MS, and their α-(1→4)-linked cyclic structures were identified by ^{1}H-NMR and ^{13}C-NMR. The ^{13}C-NMR chemical shifts of these four new LR-CDs were also elucidated and compared with those of other LR-CDs, and their structures were predicted. Our results suggest that CGTase can produce LR-CDs composed of up to about 20 GUs under standard reaction conditions.

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 preparation and purification steps.

Apparatus and Columns for Preparative and Analytical Methods The HPLC equipment used for purification and determination of purity has been described previously.[7)] A Senshu Pak ODS-1251-SS (250 mm×4.6 i.d., Senshu Kagaku, Tokyo) was used for analytical chromatography as an octadecyl silica (ODS) column and an Asahipak NH2P-50 (250 mm×4.6 i.d., Showa Denko, Tokyo) was used for both preparative and analytical chromatography as an amino (NH_2) column. The column temperature was held constant in a water bath or a column oven.

Isolation and Purification of ν-, ξ-, o- and π-CD The procedure for preparation of LR-CD mixtures has been described previously.[5)] For the isolation and purification of each LR-CD, the mixture was roughly separated into 4 fractions by HPLC on a preparative ODS column and the second (HO-2) of the fractions thus obtained was further separated into 4 fractions by HPLC on a semi-preparative NH_2 column as described previously.[7)] Fi-

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nally, *v*-CD (NH-4-3), *ξ*-CD (NH-4-4), *o*-CD (NH-4-5) and *π*-CD (NH-4-6) were purified by HPLC on the analytical Asahipak NH2P-50 column from the last of the fractions (NH-4) obtained from the semi-preparative NH_2 column.

Purity of *v*-, *ξ*-, *o*- and *π*-CD by HPLC The purities of these CDs were determined by HPLC on the ODS and NH_2 columns. HPLC was performed under the following conditions: (1) column, Senshu Pak ODS-1251-SS; eluent, methanol–water (5 : 95); flow rate, 0.8 ml/min; column temperature, 25 °C, and, (2) column, Asahipak NH2P-50; eluent, acetonitrile–water (55 : 45); flow rate, 0.7 ml/min; column temperature, 20 °C.

Characterization of *v*-, *ξ*-, *o*- and *π*-CD by Mass and NMR Spectrometry FAB-MS spectra were measured in positive-ion mode with an SX-102A mass spectrometer (JEOL, Tokyo) using Magic Bullet as the matrix. The acceleration voltage was 7 kV. ^{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 are given in *δ*-units (ppm) downfield from the signal of external tetramethylsilane [$(CH_3)_4Si$].

Results and Discussion

Isolation and Purification of *v*-, *ξ*-, *o*- and *π*-CD Figure 1 shows the chromatogram of fr. NH-4 obtained by HPLC on the analytical Asahipak NH2P-50 column. This chromatogram suggested that four components with longer retention times than *μ*-CD in fr. NH-4 were larger CD because generally the elution sequence with an aminopropyl-bonded silica column and acetonitrile–water system follows the order of the number of GUs,[14] and the Asahipak NH2P-50 column had almost the same elution sequence. *v*-CD (NH-4-3), *ξ*-CD (NH-4-4), *o*-CD (NH-4-5) and *π*-CD (NH-4-6) were purified by HPLC on the analytical Asahipak NH2P-50 column from fr. NH-4, as shown in Fig. 1.

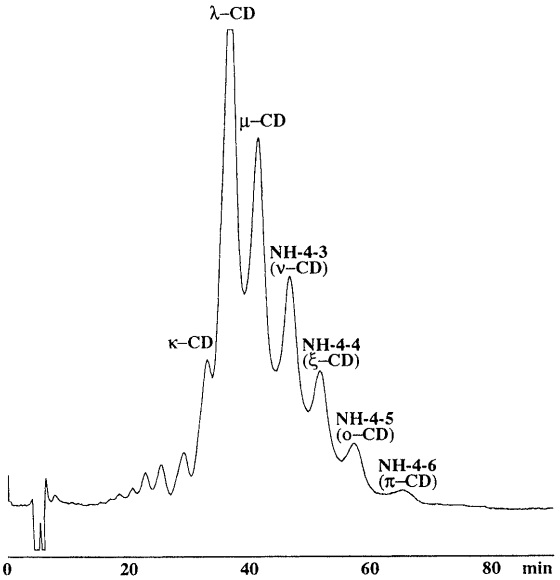

Fig. 1. Chromatogram of Fr. NH-4 on an Analytical Asahipak NH2P-50 Column

Conditions: column, Asahipak NH2P-50; eluent, CH_3CN–H_2O (58 : 42); flow rate, 0.7 ml/min; column temperature, 20 °C.

Purity of *v*-, *ξ*-, *o*- and *π*-CD Each LR-CD was analyzed by HPLC on an ODS column and an NH_2 column. The purity of *v*-, *ξ*- and *o*-CD was >98% purity. On the other hand, *π*-CD was obtained with only 90% purity. The remaining 10% appeared to be *o*-CD from the results of HPLC and FAB-MS. We obtained about 17.7 mg of *v*-CD, about 9.9 mg of *ξ*-CD, about 5.0 mg of *o*-CD and about 2.0 mg *π*-CD, and yields were 1.6×10^{-4}, 9.1×10^{-5}, 4.6×10^{-5} and 1.8×10^{-5}%, respectively based on the starting amount of CD powder, 10.88 kg.

Characterization of *v*-, *ξ*-, *o*- and *π*-CD by HPLC, FAB-MS and NMR To determine the elution sequence on the NH_2 column, the four freshly prepared LR-CDs were subjected to HPLC under the same conditions. Figure 2 shows the chromatogram of four new LR-CDs on the analytical Asahipak NH2P-50 column. The elution sequence followed the number of GUs indicating that the number of units increased in the following order: *v*-CD<*ξ*-CD<*o*-CD<*π*-CD.[14]

Each FAB-MS spectrum gave the following: *m/z* 2940.4 $[M+Na]^+$ from *v*-CD, *m/z* 3102.6 $[M+Na]^+$ from *ξ*-CD, *m/z* 3265.0 $[M+Na]^+$ from *o*-CD and *m/z* 3426.5 $[M+Na]^+$ from *π*-CD. These findings were in agreement with the calculated molecular weights of *v*-CD (Calcd for $(C_6H_{10}O_5)_{18}$: 2916.951), *ξ*-CD (Calcd for $(C_6H_{10}O_5)_{19}$: 3079.004), *o*-CD (Calcd for $(C_6H_{10}O_5)_{20}$: 3241.057) and *π*-CD (Calcd for $(C_6H_{10}O_5)_{21}$: 3403.109), and indicated that the four new LR-CDs were composed of 18, 19, 20 and 21 GUs, respectively.

The ^{1}H-NMR, ^{13}C-NMR and two-dimensional ^{1}H–^{13}C correlation (H, C COSY) NMR spectra of the four LR-CDs were measured with a JNM-LA500 spectrometer at 50 °C in deuterium oxide. Figure 3 shows the ^{1}H–^{13}C correlation (H, C COSY) NMR spectrum of *o*-CD as a representative example. Their assignments could be made from the two-dimensional ^{1}H–^{13}C correlation (H, C COSY) NMR spectra. The spectra of *v*-, *ξ*-, *o*- and *π*-CD showed six clear and distinct single peaks attributable to equivalent GUs in the each ^{13}C-

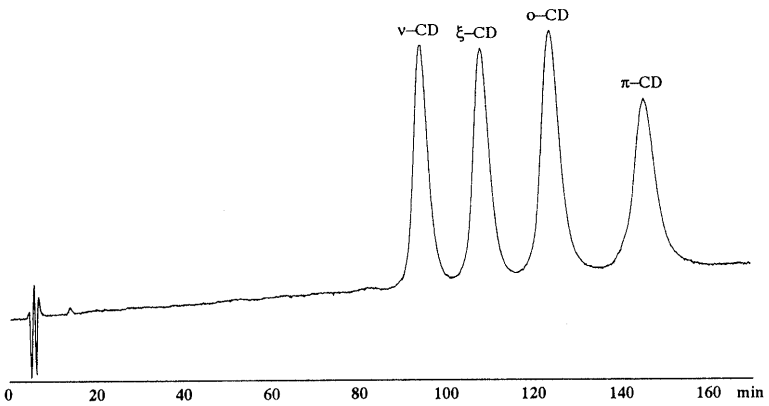

Fig. 2. Elution Profile of *v*-, *ξ*-, *o*- and *π*-CD on an Analytical Asahipak NH2P-50 Column

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

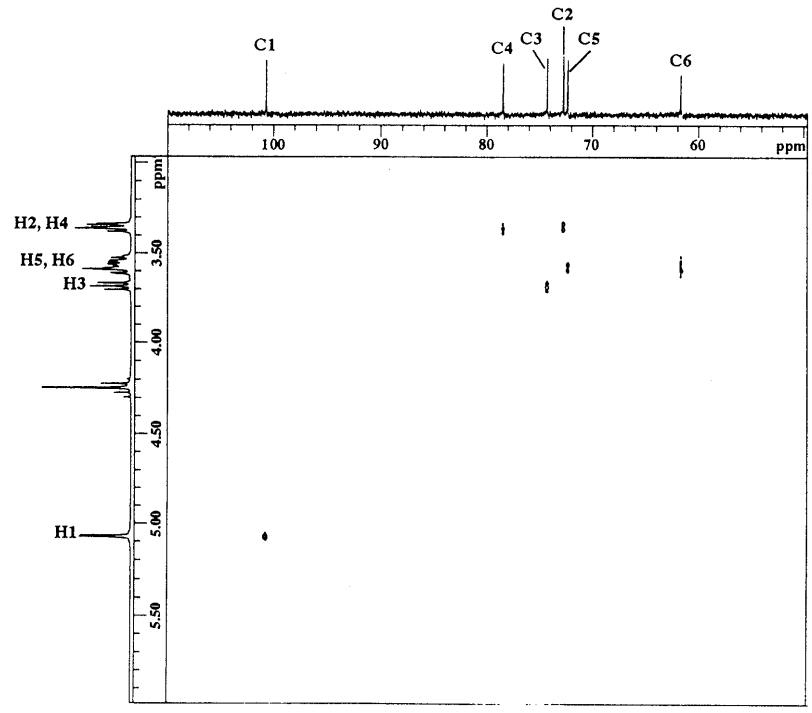

Fig. 3. ^{1}H–^{13}C COSY NMR Spectrum of *o*-CD
Solvent, deuterium oxide; temperature, 50 °C.

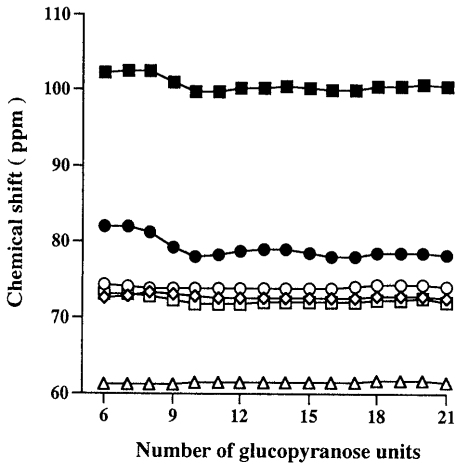

Fig. 4. Variation in ^{13}C Chemical Shifts of CDs with Number of Glucopyranose Units
■, C1; ◇, C2; ○, C3; ●, C4; □, C5; △, C6.

NMR spectrum. Figure 4 shows the variation of chemical shift values at each carbon in sixteen kinds of CDs. We have already reported that the chemical shift values at C1 and C4 in LR-CDs were shifted upfield in comparison with conventional CDs and these differences were most likely due to the distorted cyclic structure of LR-CDs.[7,8] The chemical shift values of each carbon in *ν*-, *ξ*-, *o*- and *π*-CD were almost the same as those of other LR-CDs except for *δ*-CD. These results suggested that *ν*-, *ξ*-, *o*- and *π*-CD would have the saddle-like structures of *ε*-[9] and *ι*-CD.[10]

In conclusion, we isolated and purified LR-CDs composed of 9 to 21 GUs, designated *δ*-CD to *π*-CD, from commercially available CD powder produced by CGTase from potato starch. The presence of these LR-CDs was confirmed by HPLC, FAB-MS and NMR. Although CGTase could also produce LR-CDs larger than *π*-CD, they were present in very small amounts, if at all, and could not be detected in this experiment. These results suggest that CGTase can produce LR-CDs containing up to about 20 GUs under standard reaction conditions; *i.e.* use of natural starch such as potato starch as the substrate and long reaction times. However, this preparation method was not efficient, because the yield of each LR-CD was very low. Fortunately, CGTase can efficiently produce such LR-CDs under different reaction conditions[13] and, furthermore, the potato D-enzyme was demonstrated to efficiently produce LR-CDs composed of more than 17 GUs.[12] These methods should be used to prepare large amounts of each LR-CD.

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References and Notes

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