Separation and Characterization of Three Positional Isomers of Dimaltosyl-β-cyclomaltoheptaose (Dimaltosyl-β-cyclodextrin)\(^{11}\)

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A mixture of maltosylcyclomaltoheptaoses (maltosyl-β-cyclodextrins, G\(_2\)-βCDs) was prepared from maltose and β-cyclodextrin (βCD) through the reverse action of Klebsiella pneumoniae pullulanase. Three positional isomers of dimaltosyl-βCD in the mixture were separated by high-performance liquid chromatography on a reversed phase column and a graphitized carbon column. Their molecular weights were measured by fast-atom bombardment mass spectrometry, and the structures were established by methylation analysis, hydrolysis with glucoamylase to the known compounds, three positional isomers of diglucosyl-βCD, and \(^{13}\)C-nuclear magnetic resonance spectroscopy.

**Key words** maltosyl-β-cyclodextrin; dimaltosyl-β-cyclodextrin; positional isomer; HPLC; reversed phase column; graphitized carbon column; methylation analysis; glucoamylolysis; FAB-MS; \(^{13}\)C-NMR

Maltosylcyclomaltoheptaoses (maltosyl-β-cyclodextrins, G\(_2\)-βCDs) have been prepared from maltose and β-cyclodextrin (βCD) by the reverse condensation reaction of debranching enzymes such as pullulanase\(^{2-4}\) or isomaltase\(^{4-6}\) and have one or more maltosyl branches linked by \(\alpha\)-1,6 glucosidic linkage to glucose residues in βCD. Dimaltosyl-βCDs [(G\(_2\))\(_2\)-βCDs] are expected, having an analogue characteristics with diglucosyl-βCDs\(^{7-9}\) to have higher solubility both in water and in organic solvents, lower hemolytic activity, and to be safer in other metabolism experiments than parent βCD and even monomaltosyl-βCD [G\(_2\)-βCD], and hence are expected to be very useful for the solubilization of water-insoluble or slightly soluble drugs.

We describe here the separation and structural analyses of three positional isomers of (G\(_2\))\(_2\)-βCD (Chart 1) in a mixture of maltosyl-βCDs which was prepared from maltose and βCD using an enzymatic reaction.

**Experimental**

**Materials** \(\beta\)CD (Ensuiso Sugar Refining) and maltose (Sanwa Denpun Kogyo) were commercial products. Pullulanase from Klebsiella pneumoniae "Pullulanase Amano 3" (3000 U/ml) and glucoamylase from Rhizopus niveus "Gurukazaimu AF" (6000 U/ml) were both commercial products (Amano Pharmaceutical). One unit of pullulanase activity is defined as the amount of enzyme causing an increase of reduction corresponding to 1 \(\mu\)mol of glucose from pullulan per minute, and one unit of glucoamylase activity is the amount of enzyme that forms 10 mg of glucose from soluble starch in 30 min at 40°C, pH 4.5. 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** Optical rotations were measured with a JASCO digital polarimeter, model DIP 360. High-performance liquid chromatography (HPLC) was performed with a JASCO 880-PU pump, a Waters 660 universal injector, and a Showa Denko SE-61 refractive index detector. The columns used were a YMC-Pack AQ-325 ODS (250 \(\times\) 10 mm i.d.) and a Hypercarb (100 \(\times\) 4.6 mm i.d.) (Shandon Scientific). HPLC analyses at constant temperature were conducted using a column oven SSC 3510C (ShinShu Scientific Co.). A Shimadzu Chromatopac C-R3A digital integrator was used for integration of peak areas. Fast-atom bombardment-mass spectrometry (FAB-MS) was performed with a JEOL JMS-DX 303 mass spectrometer using xenon atoms having a kinetic energy equivalent to 6 keV at an accelerating voltage of 3 kV. The mass marker was calibrated with perfluoroalkylphosphazene (Ultra Mark), and glycerol was used as the matrix. \(^{13}\)C-Nuclear magnetic resonance (\(^{13}\)C-NMR) spectra (125.65 MHz) of 2-3% solutions in D\(_2\)O were recorded at ambient temperature with a JEOL GXS-300 spectrometer. Chemical shifts were expressed in ppm downfield from the signal of Me\(_2\)Si using 1,4-dioxane (67.40 ppm) as the external standard.

**Preparation of a Mixture of Maltosyl-βCDs** Maltose (25 g) was dissolved in 5 ml of 25 mm acetate buffer (pH 6.0) at 90°C and βCD (5 g) was added to the solution. After cooling to 55°C, 0.6 ml of Pullulanase Amano 3 (3000 U/ml) was added, the mixture was incubated for 72 h, and inactivated by heating. Using a C\(_18\)-bonded silica column (1000 \(\times\) 24.4 mm i.d., Organosil) with water, the remaining maltose in the reaction mixture was removed, and then a mixture of maltosyl-βCDs was eluted from the column with ethanol-water (10:90 v/v) at a flow rate of 15 ml/min, concentrated, and lyophilized (4.8 g).

**Methylation Analysis** Methylation of dimaltosyl-βCDs was performed by the method of Preece\(^{10}\) with 2,6-di-(tert-butyl)-pyridine and methyl trifluoromethane-sulfonate in trimethyl phosphate. The products were hydrolyzed, converted to their alditol acetates, and then analyzed with a Hitachi gas chromatograph model 063 fitted with a flame-ionization detector.

**Hydrolysis of Dimaltosyl-βCDs with Glucoamylase** (G\(_2\))\(_2\)-βCD sample

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**Chart 1**

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(5 mg) and glucoseisomerase (37.8 U) were dissolved in 0.5 ml water, and the solution was incubated at 40 °C for 24 h. The reaction mixture was heated at 100 °C for 5 min and then the denatured enzyme was removed by filtration through a 0.2-μm membrane filter. The hydrolyzate, that is, diglucosyl-β CD was characterized by HPLC.

**Results and Discussion**

**Separation**  Figure 1 shows a chromatogram of a mixture of maltosyl-β CDs prepared from maltose and β CD through the reverse action of Klebsiella pneumoniae pullulanase. The components corresponding to peaks 1 and 2 were separated. Although two components should be contained in the peak 1, separation of those was impossible on the reversed column. A new type column packed with graphitized carbon made the separation possible (Fig. 2). The graphitized carbon column, Hypercarb has been demonstrated to have unique ability of resolving isomeric and closely related compounds, and has been used for the analyses of samples such as pharmaceutical products, amino acids, peptides, and phenols. However, it has some disadvantages, e.g., a small loading capacity (less than 1 mg) and a marked tendency of tailing owing to the main mechanism of retention on the column, namely adsorption. Consequently, although this column is very useful for the analysis, it is hardly used for preparative HPLC. In this work isolation of component B corresponding to peak 1B was extremely difficult, though isolation of the first eluting component A was relatively easy. In order to exclude any trace contamination of A in the fraction of B, many repetitive chromatographic separations of the latter fraction were necessary.

The ratios of G2− and (G2)2−/βCDs were 7:3, and those of three isomers of (G2)2/βCD, A, B, and C corresponding to peak 2 in Fig. 1 were 3.4:4.2:1.0, as measured from the chromatograms of Figs. 1 and 2.

**Determination of Molecular Weight** The molecular weights of A, B, and C confirmed by FAB-MS were all 1782, that is, comprised of 11 glucose units. Moreover, two peaks for fragment ions [M−G−H]− (m/z 1619) and

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**Fig. 1. Chromatogram of a Mixture of Maltosyl-β CDs on ODS Column**

1, dimaltosyl-β CD (A and B); 2, dimaltosyl-βCD (C); 3, monomaltosyl-βCD; 4, βCD. Chromatographic conditions: column, YMC-Pack AQ-323 (250 × 10 mm i.d.); eluent, CH3OH−H2O (10:90); flow rate, 2.5 ml/min; temperature, 50°C.

**Fig. 2. Separation of Two Positional Isomers of Dimaltosyl-β CD on Graphitized Carbon Column**

1A, dimaltosyl-β CD (A); 1B, dimaltosyl-β CD (B). Chromatographic conditions: column, Hypercarb (100 × 4.6 mm i.d.); eluent, CH3CN−H2O (16:84); flow rate, 1 ml/min; temperature, 50°C.

**Fig. 3. FAB-MS Spectra of Compounds A, B, and C in Negative Mode**
TABLE I. Methylation Analyses of Compounds A, B, and C

<table>
<thead>
<tr>
<th>Product</th>
<th>$t_k$ (min)</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol</td>
<td>6.4</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1,4,5-Tris-O-acetyl-2,3,6-tri-O-methyl-D-glucitol</td>
<td>18.0</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>1,4,5,6-Tetra-O-acetyl-2,3-di-O-methyl-D-glucitol</td>
<td>38.4</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Gas chromatographic conditions: column, 0.3% OV-275—0.4% GEXF-1150 on Shimalite W (AW-DMCS), 80—100 mesh (2 mm x 3 mm i.d.), column temperature, 160°C; carrier gas and flow rate, N₂, 30 ml/min.

Fig. 4. $^{13}$C-NMR Spectra of Compounds A, B and C Measured in D$_2$O at 125.65 MHz

C: the carbon atom of the ring D-glucuronic unit. C and C': the carbon atom of the branched unit. C-6': the carbon atom of the branch point.

$[M-2G-H]^- (m/z 1457)$ were observed in all spectra of A, B, and C (Fig. 3). These fragment ions must be formed through a single cleavage of the side-chain (primary fragments). Thus it was proved that the side-chains of A, B, and C were each two maltosyl residues, and not 6'-maltosylmaltosyl residues from which two other kinds of primary fragments, $[M-3G-H]^- (m/z 1295)$ and $[M-4G-H]^- (m/z 1133)$ could be generated.  

**Methylation Analysis** The results of methylation analyses of A, B, and C indicated that all of them are 6',6''-di-O-maltosyl-β-CDs (Table I).

**Hydrolysis with Glucoamylase** Three isomers of di-maltosyl-βCD (A, B, and C) were hydrolyzed with glucoamylase to corresponding diglucosyl-βCDs whose structures had been established by HPLC analysis of partial hydrolyzates, $^{13}$C-NMR spectroscopy, and chemical synthesis.  

The results show that A, B, and C are 6',6''-6',6''-di-O-maltosyl-β-CDs, respectively. $^{13}$C-NMR Spectroscopy Figure 4 shows $^{13}$C-NMR spectra of dimaltosyl-βCDs (A, B, and C). Assignments of signals in the spectra could be made by analogy with those in the spectra of diglucosyl-βCDs. The assignments of C-6 signals were confirmed by the insensitive nuclei enhanced by polarization transfer (INEPT) method,  

Using $\Delta = 3.4 J$. The large downfield shift of two C-6 signals indicates that the side-chain maltose residues are attached to oxygenes on these carbon atoms. The ratios in the signal intensities of CD ring C-6 (at δ ca. 61.3), side-chain C-6 (at δ ca. 61.5, C-, and C''-6) and branch-point C-6 (at δ ca. 68.1, C-6') were 5:4:2. Those of ring C-4 (at δ 81.7—82.7), side-chain C-4 involved in α(1→4)-linkage (at δ ca. 78.8, C-4) and at nonreducing end (at δ ca. 70.3, C''-4) were 7:2:2, and those of ring C-1 (at δ 102.4—102.7), side-chain C-1 involved in α(1→4)-linkage (at δ ca. 100.9, C-1') and in α(1→6)-linkage (at δ ca. 99.5, C-1') were also 7:2:2. In the spectrum of C, C-6', C-4', and C-1' signals are each split into two peaks; the C-6' signal of A is also split and in the expanded spectrum of A, C-4' and C-1' signals were each slightly split into two peaks. Those signals of B, except for the very slightly split C-6' signal were observed as single peaks, respectively, even in the expanded spectrum. These facts indicate the difference of magnitude of interaction between two side-chain maltosyl residues in each molecule of A, B, and C.

**Optical Rotation** The $[\alpha]_{D}^{20}$ values in H$_2$O were +170.0° (c=0.3) for A, +170.0° (c=0.3) for B, and +171.7° (c=0.3) for C.

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**References and Notes**

1) This work was presented at The 9th Cycloextrin Symposium, Tsukuba, November 1990.
11) Application notes, Shandon Scientific Ltd., Chadwick Road, Astmoor, Runcorn, Cheshire WA7 1PR, England.