Note

Synthesis of Branched α -Cyclodextrin Carrying a Side Chain Longer than Maltose Using *Bacillus acidopullulyticus* Pullulanase

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Branched cyclodextrins (Br-CDs) are synthesized from amylopectin using cyclodextrin glucanohydrolase (EC 3.2.1.19),^{1,2)} and from CDs and maltose or maltosyl-fluoride using isoamylase (EC 3.2.1.68)^{3,4-6)} or pullulanase (EC 3.2.1.41).^{3,4,7)} Their solubility is higher than that of corresponding CDs without loss of their hydrophobicity, stabilizing labile compounds, and masking taste, flavor, or odor. CD application will be expanded to the fields of foods and pharmaceuticals if Br-CD can be economically produced industrially. Single Br-CDs that carry a longer side chain the G_2 are also expected to be suitable substrates to analyze the activity of debranching amylase because they have a single attack point of the enzyme and no reducing end.

There has been no report on synthesis of Br-CDs with longer side chain than G_2 besides Hizukuri and his coworkers' reports,^{5,6)} in which G_3 -, G_4 -, and G_5 - α -CDs were synthesized in 10~20% yields using PA-lase and identified by ¹³C-NMR and methylation analyses. We have reported the enzymic and large scale synthesis of G_2 - α -, G_2 - β -, and G_2 - γ -CD in 45~48% yields, and their molecular properties.⁸⁾ This report describes the synthesis of G_3 -, G_4 -, G_5 -, and G_6 - α -CD in yield higher than 20%

from a concentrated mixture of G_3 , G_4 , G_5 , or G_6 and α -CD using the reverse reaction of BA-Pase, and identification of these Br-CDs using hydrolysis by KP-Pase, methylation analyses, and ¹³C-NMR analyses.

The reaction conditions were based on the synthesis of G_2 - α -CD⁸⁾; the reaction mixture, containing 0.16~0.24 M α -CD, 0.8~1.9 M maltooligosaccharide and 16~23 units/ml of BA-Pase* dissolved in 50 mM acetate buffer at pH 5.0, was incubated at 60°C for 70~96 hr. BA-Pase activity was measured as described previously⁸⁾ and one unit of enzyme activity is defined as the amount of enzyme hydrolyzing 1 μ mol of (α 1 $\rightarrow$ 6) linkage in pullulan per min under these conditions.⁸⁾ The reaction was stopped by changing the pH from 5.0 to 7.0 with 1 N NaOH and autoclaving at 110°C for 15 min. Conversion yields to G_3 -, G_4 -, G_5 -, and G_6 - α -CD from α -CD were 35, 33, 28, and 22% respectively.

The reaction mixture was diluted 2-fold with distilled water to decrease its viscosity, and put onto a column of Preparative C18 ODS (90 g, Waters Co., Ltd.). After the column was thoroughly washed with distilled water to remove non-cyclic oligosaccharides, the α -CD and Br- α -CD synthesized were eluted with 30% methanol. The fractions containing CDs were purified using gel filtration on a Toyopearl HW-40S column (10 $\times$ 100 cm $\times$ 2, Toso Co., Ltd.)⁸⁾ and HPLC on a YMC packed column ODS-50 (20 $\times$ 250 mm, Yamamura Chemical Laboratory Inc.) with a solvent of methanol-water (1:9, v/v).

Each purified G_3 -, G_4 -, G_5 -, and G_6 - α -CD showed a single peak in HPLC on a YMC packed column A-612 (NH_2) (6 $\times$ 150 mm, Yamamura Chemical Laboratory Inc.) with a solvent of acetonitrile-water (55:45 v/v) at a flow rate of 1.5 ml/min (Fig. 1), and on a YMC packed column A-312 ODS (6 $\times$ 150 mm, Yamamura Chemical Laboratory Inc.) with a solvent of methanol-water (8:92 v/v) at a flow rate of 1.5 ml/min (data not shown). There is a relationship between the number of glucose residues (N_g) in a side chain linked to the ring of α -CD and the retention time R_t of Br-CD; a plot of log R_t against N_g gave a straight line (NH_2 column) or a sine curve (ODS column) as shown in Fig. 2. Yields of G_3 - α -CD, G_4 - α -CD, G_5 - α -CD and G_6 - α -CD were 1.5~1.6, 1.5~1.6, 1.8~1.9, and 1.0~1.1 mmol from 10 mmol of α -CD, respectively, and each purity was greater than 99.9%.

Each purified Br- α -CD was non-reducing, and was hydrolyzed by KP-Pase into the corresponding maltooli-

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Abbreviations: G_2 , maltose; CD, cyclodextrin; α -CD, α -cyclodextrin; β -CD, β -cyclodextrin; γ -CD; γ -cyclodextrin; Br-CD, branched cyclodextrin; Br- α -CD, branched α -cyclodextrin; G_2 - α -CD, maltosyl(α 1 $\rightarrow$ 6) α -cyclodextrin; G_3 - α -CD; maltotriosyl(α 1 $\rightarrow$ 6) α -cyclodextrin; G_4 - α -CD, maltotetraosyl(α 1 $\rightarrow$ 6) α -cyclodextrin; G_5 - α -CD, maltopentaosyl(α 1 $\rightarrow$ 6) α -cyclodextrin; G_6 - α -CD, maltohexaosyl(α 1 $\rightarrow$ 6) α -cyclodextrin; BA-Pase, *Bacillus acidopullulyticus* pullulanase; KP-Pase, *Klebsiella pneumoniae* pullulanase; PA-lase, *Pseudomonas amyloclavata* isoamylase.

* BA-Pase: Cold acetone (-20°C) was added to Promozyme 200L (Novo Industri A/S, Denmark) (1:1, v/v) to precipitate BA-Pase. This partially purified enzyme, which had a specific activity of 88 units/mg of protein, was used for synthesis of Br-CDs.

Table I. CHEMICAL SHIFTS OF SIGNAL FOR C-1, C-4 AND C-6 IN ^{13}C -NMR SPECTRA OF SOLUTION OF BRANCHED α -CD IN D_2O

Branched α -CD	C-1	C-1'	C-1'', 1'''	C-4	C-4'''	C-4', 4''	C-6, C-6', 6'', 6'''	C-6a
G ₂ -	101.2	97.4	98.8	80.0~80.5	68.1	76.2	59.3	66.1
G ₃ -	101.4	98.6	99.9~100.1	81.2~81.7	69.5	77.0, 78.1	60.5	67.3
G ₄ -	101.4	98.6	99.7~100.1	81.2~81.7	69.4	77.1, 78.0	60.5	67.4
G ₅ -	101.4	98.6	99.6~100.0	81.2~81.8	69.4	77.2, 78.0	60.5	67.4
G ₆ -	101.4	98.6	99.6~100.0	81.2~81.8	69.4	77.1, 78.0	60.5	67.4

C-1, C-4 and C-6: the carbon atoms of the ring residues. C-6a: the carbon atom of branched point. C-1', C-4' and C-6': the carbon atoms of the side-chain residue linked to the main ring. C-1'', C-4'' and C-6'': the side-chain residues with HO-4 unsubstituted. C-1''', C-4''' and C-6''': the carbon atoms of the other side-chain residues.

Fig. 1. HPLC of α -CD and Br- α -CDs on NH_2 -bonded Silica Column.

Column, YMC packed column A-612 NH_2 (6×150 mm); detector, Erma ERC-7521 RI detector; eluent, acetonitrile-Water (55:45, v/v); flow rate, 1.5 ml/min. s, H_2O ; 0, α -CD; 1, G₁- α -CD; 2, G₂- α -CD; 3, G₃- α -CD; 4, G₄- α -CD; 5, G₅- α -CD; 6, G₆- α -CD.

gosaccharide and α -CD at a molar ratio of 1:1. SIMS (Secondary Ion Mass Spectrometry) and ^{13}C -NMR analysis of Br- α -CDs were done as described previously.⁸⁾ The results are as follows. 1) From the mass number of each MH^+ ion of Br- α -CD measured by SIMS, the molecular weights of G₃- α -CD, G₄- α -CD, G₅- α -CD, and G₆- α -CD were 1,458 ($=162 \times 9$), 1,620 ($=162 \times 10$), 1,782 ($=162 \times 11$) and 1,944 ($=162 \times 12$). 2) The ^{13}C -NMR spectra of each Br- α -CD was assigned as shown in Table I. The C-1 resonances of glucose residues of the main ring (C-1 in Table I), side-chain residues linked to the main ring (C-1'), and the other side-chain residues (C-1'' and C-1''') of G₂-, G₃-, G₄-, G₅-, and G₆- α -CD appeared separately at

Fig. 2. Relationship between $\log R_t$ and N_g for α -CD and Br- α -CDs in HPLC on HN_2 -bonded Silica Column and ODS Column.

Column, YMC packed column A-612 NH_2 (6×150 mm) (—●—) and YMC packed column A-312 ODS (6×150 mm) (—○—); eluent, acetonitrile-Water (55:45, v/v) for NH_2 column and methanol-Water (8:92, v/v) for ODS column; detector, Erma ERC-7521 RI detector; flow rate, 1.5 ml/min.

$\delta \sim 101$, ~ 98 , and ~ 100 , respectively, and the relative intensities of these signals were 6:1:1 (G₂- α -CD), 6:1:2 (G₃- α -CD), 6:1:3 (G₄- α -CD), 6:1:4 (G₅- α -CD), and 6:1:5 (G₆- α -CD). 3) The coupling constant ($J_{\text{C-1,H-1}}$) of each C-1 atom was ~ 170 Hz, so the configuration of C-1 in the glucosyl residue linked to α -CD ring was α^2 , as well as that of C-1 in the α -CD ring. From the results above, Br- α -CDs obtained were found to be α -CDs having a single side-chain and a ($\alpha 1 \rightarrow 6$) link per molecule; they were identified as G₃- α -CD, G₄- α -CD, G₅- α -CD and G₆- α -CD.

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