Cyclodextrin-Dialdehyde Prepared by Periodate Oxidation

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Dialdehyde derivative of cyclodextrin (dial-CD) was prepared by oxidation with sodium metaperiodate, because the oxidative cleavage with periodate would theoretically provide 2',3'-dialdehyde derivatives of the α -1,4-linked glucans. The α -CD oxidized with equimolar periodate (dial- α -CD I) was separated from the unoxidized native α -CD by column chromatography on Sephadex G-15, where the peak of dial- α -CD I was eluted faster than the native α -CD and corresponded to the reducing power. Since the hydrated forms of dial-CD gave no characteristic spectral pattern of the dialdehyde groups, the structure of the dial-CD was deduced by IR and ^{13}C -NMR spectral analysis for a dicarboxylate derivative of the dial- α -CD. The dial- α -CD I has an average of 3.5 glucose residues oxidized into the dialdehyde structure. Among the possible 13 isomers of the dial- α -CD, isomers IA, IIAB, IIAC, IIIABC, and IIIABD were the predominant forms, which were identified by the measurement of maltooligosaccharides derived from the dial- α -CD I by NaBH_4 reduction and acid treatment. Moreover, the reactivity of the dial- α -CD I was demonstrated by adduct formation with lysine and glycine, which was analyzed by gel filtration patterns on a Sephadex G-15 column.

Cyclodextrins (CD) of α -, β -, and γ -forms are nonreducing oligosaccharides containing α -1,4-linked D-glucose, and have several unique characteristics.¹⁾ For example, various CD derivatives were recently investigated for the sake of wide development of the chemical construction of enzyme mimics.²⁾ Moreover, many forms of CD derivatives were synthesized because of their inclusion-complex forming function with drugs and water-insoluble compounds.³⁾

Oxidation of starch by various oxidizing agents has been extensively studied for practical applications.⁴⁾ Periodate oxidation is useful for the structural analysis of polysaccharides. Since CD has neither reducing nor nonreducing terminal D-glucose molecules, a controlled periodate oxidation would be effective to introduce a dialdehyde group at the C-2, C-3-*trans*-diol position of D-glucose residues in the

CD molecule. The stoichiometry of the reaction (the number of moles of periodate consumed per glucose residue) and no significant excessive overoxidation were reported for β -CD by French and McIntire.⁵⁾ The periodate oxidized dialdehyde derivatives (2',3'-*cis*-diol) of ribonucleotides have been shown to react with lysine residues of proteins to form Schiff base adducts.^{6,7)} Therefore, it is very interesting to prepare the CD molecules introduced a dialdehyde structure, which is highly reactive with proteins.

In this study, we describe the preparation and the structural analysis of periodate-oxidized CD (dial-CD). The major interest in the dial-CD is the application as enzyme inhibitors or labeling reagents based on the high reactivity of the aldehyde group with amino groups in the enzyme molecule.

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MATERIALS AND METHODS

Materials. α -, β -, and γ -Cyclodextrins (CD) were the products of Nihon Shokuhin Kako Co., Ltd. Methyl 4,6-benzylidene- α -D-glucopyranoside was provided by Dr. Tetsuya Murayama in our department.

Periodate oxidation. α -, β -, and γ -CD, 30 mM, were mixed with sodium metaperiodate (30 mM) and oxidized at 30°C in the dark. The CD oxidized with one mol of periodate to one mol of CD referred to as dial-CD I. Alternatively CD was oxidized with a 2 to 12-fold molar excess of sodium metaperiodate, and these products are referred to as dial-CD II to dial-CD XII, respectively. Methyl 4,6-benzylidene α -D-glucopyranoside (30 mM) was oxidized with 30 mM sodium metaperiodate at 30°C in the dark with shaking. Oxidation was monitored by UV at 222.5 nm. The reaction was stopped by the addition of 50 mM ethylene glycol and the resulting reducing power was measured by the Nelson-Somogyi method.⁸⁾ The dial-CD was purified with column chromatography on Sephadex G-15.

Preparation of α -CD-dicarboxylate. The dialdehyde group of the periodate-oxidized α -CD (dial- α -CD I) was further oxidized to carboxylic acid by the hypiodite method.⁹⁾ The reaction mixture was put on a column of Bio-Gel P-2 and the void volume fraction was collected as the α -CD-dicarboxylate (α -CD-COOH). The pooled fraction was treated with AG11A8 (retardion resin, Bio-Rad) and evaporated to dryness. Carboxylate derivatives of maltose was prepared in the same way.

Analytical method. The reducing power of dial-CD was measured by the Nelson-Somogyi method⁸⁾ or a fluorescent reagent, BEC (borate ethanolamine complex) as described previously.¹⁰⁾ Total sugar was measured by the phenol sulfuric-acid method.¹¹⁾ Measurement of formic acid was done by the acetylacetone method.¹²⁾ Unoxidized D-glucose residues were measured as follows: dial-CD was hydrolyzed with 1 M H_2SO_4 at 100°C for 6 hr. The hydrolyzate was neutralized with 1 M NaOH solution and the amount of remaining D-glucose was measured by a glucose oxidase method (Glucose B-TEST, Wako Pure Chemicals). The dial- α -CD I (15 mg) was reduced with $NaBH_4$ (15 mg) at 28°C overnight, then acidified (pH 1.0) with 1 M HCl. Inorganic salts were removed with Amberlite IR-120 and IRA-410 resins. Paper chromatography was done with a solvent system of *n*-butanol-*n*-propanol-water (3:5:4 by volume). For the separation of maltooligosaccharides derived from the dial- α -CD I, 65% *n*-propanol was used as a solvent, thrice ascending at 60°C. Spots were detected by the alkaline silver nitrate dip procedure.¹³⁾ IR spectra were recorded with a JASCO IR-810 infrared spectrometer for KBr discs. ^{13}C -NMR spectra were recorded with a JEOL FX-100 spectrometer, each CD derivative (20~40 mg) being dissolved in deu-

terium oxide (0.3 ml). Amino acids were measured by the fluorescent method using *o*-phthalaldehyde.¹⁴⁾

RESULTS

Preparation of dial-CD

To prepare the dialdehyde compound, α -CD was oxidized with sodium metaperiodate at 30°C in the dark. As shown in Fig. 1, oxidation with an equimolar (dial- α -CD I) to 3-fold molar excess (dial- α -CD III) of sodium metaperiodate reached a maximum in 6 to 8 hr. Consumption of periodate was clearly proportional to the ratio of sodium metaperiodate to α -CD. For β - and γ -CD (dial- β -CD I and dial- γ -CD I), the oxidation progressed similarly (data not shown). Comparison of the periodate consumption of CD with the authentic methyl 4,6-benzylidene α -D-glucopyranoside, in which only the linkage between C-2 and C-3 was susceptible to oxidation, showed that the reactions of CD with periodate were highly stoichiometric. Production of the aldehyde group was confirmed by the

FIG. 1. Periodate Oxidation of α -CD and Benzylidene Glucoside.

α -CD (30 mM) was oxidized with 30 mM ($\bullet$, $\circ$), 60 mM ($\blacktriangledown$, $\triangledown$), and 90 mM ($\blacksquare$, $\square$) sodium metaperiodate at 30°C in the dark. Methyl 4,6-benzylidene- α -D-glucopyranoside (30 mM) was oxidized with 30 mM ($\blacklozenge$, $\lozenge$) at 30°C in the dark with shaking. Consumption of metaperiodate ($\bullet$, $\blacktriangledown$, $\blacksquare$, $\blacklozenge$) and production of reducing power ($\circ$, $\triangledown$, $\square$, $\lozenge$) were measured at the indicated times. Time scales corresponded to the case of α -CD (below) and benzylidene glucoside (above).

* The amount of $NaIO_4$ consumed (mol) was represented per mol of α -CD or benzylidene glucoside.

reducing power, which was measured by the Nelson-Somogyi method (Fig. 1). The amounts of reducing power for the dial- α -, β -, and γ -CD I were at the same level, and for the dial- α -CD I, II, and III were roughly proportional to the concentration of sodium metaperiodate. Above this ratio the reducing power became stationary, for dial- α -CD VI, and then decreased slightly (Fig. 2). Measurement of the free aldehyde by the acetylacetone method¹²⁾ showed that the reaction mixture of dial- α -CD I to IV contained no significant amounts of formaldehyde (less than 0.01 mol/mol α -CD). Since the pH value of the reaction mixture was neutral, formic acid production was negligible. Thus, the over-oxidation of dial-CD would be minimal under

the above conditions.

Measurement of the numbers of unoxidized D-glucose molecules by complete acid hydrolysis followed by the glucose oxidase method gave a value of 4.68, which indicated almost one-sixth of the D-glucose molecules in the isolated dial- α -CD I fraction were oxidized.

Characterization of dial- α -CD I

Dial- α -CD I was separated from the unoxidized native α -CD by a column of Sephadex G-15 (Fig. 3a). The dial-CD was recovered in the first peak, which corresponded to the reducing power. The second peak (native α -CD) gave no response in the reducing power assay, and gave a spot with the same R_{Glc} value as the authentic native α -CD. The ratio of the first and second peaks was about 1:2 (32.6% and 67.4%). The purified dial- α -CD I gave a single peak of total sugar and reducing power upon rechromatography on a Sephadex G-15 column (Fig. 3b). Quantitative paper chromatographic analysis of the purified dial- α -CD showed that dial- α -CD (R_{Glc} 0.51) moved slightly above the native α -CD (R_{Glc} 0.37) and corresponded with the peak of reducing power, which was measured after the elution of sugars from the filter paper with water (data not shown).

Then the purified dial- α -CD was analyzed by IR and ^{13}C -NMR spectroscopy. Since the dialdehyde structure of dial- α -CD tends to have hydrated forms, no significant difference

FIG. 2. Effects of NaIO_4 Concentration on the Production of Reducing Power and Formaldehyde.

α -CD was oxidized with the indicated concentrations of NaIO_4 and the formaldehyde (●) and reducing power (○) were measured. The scale for formaldehyde indicated at the y-axis was multiplied by 10^2 (0.038 to 3.8×10^{-2}).

FIG. 3. Elution Patterns of Dial- α -CD from the Sephadex G-15 Column (2.3×75 cm).

(a): The reaction mixture (30 mg in 1.0 ml) was put on the column, and total sugar (▽) and reducing power (●) were monitored after elution with water. Tubes No. 53~74 were pooled, concentrated, and chromatographed again (b) on the Sephadex G-15 column (2×17 cm).

FIG. 4. IR and ¹³C-NMR Spectra of α -CD and Its Derivatives.

(a): IR spectra of native α -CD (I), dial- α -CD (II), and α -CD-COOH (III).

(b): ¹³C-NMR spectra of I, II, and III.

in the IR spectra between dial- α -CD and native α -CD was observed (Fig. 4a). ¹³C-NMR showed new resonances corresponding to the C-1 (102.3~102.8 ppm) besides several peaks originating from the dialdehyde structure (Fig. 4b). However, most of the new resonances could not be assigned because of the very complex structure of the hydrated forms of dial- α -CD.

Dicarboxyl derivative of dial-CD I

To obtain further spectral evidence for the dialdehyde structure of the dial-CD I, the dial- α -CD I was further oxidized to the carboxyl derivative by the hypiodite method.⁹⁾ The dicarboxyl derivative of dial- α -CD (α -CD-COOH) was isolated by a column of Bio-Gel

FIG. 5. Elution Pattern of α -CD-Dicarboxylate (α -CD-COOH) from the Bio-Gel P-2 Column (1.5 $\times$ 33 cm).

Total sugar (∇) was monitored by the phenol sulfuric-acid method. Positions of void volume (V_o), exclusion volume (V_i), and the elution pattern of α -CD ($\bullet$) are also indicated by arrows.

P-2 (Fig. 5). Like hypiodite-oxidized maltose (maltobionic acid), α -CD-COOH was eluted at the void volume of this column. The second peak contained a small amount of the unreacted dial-CD (tube No. 37~45) and unknown non-carbohydrate compounds (tube No. 45~55), the latter fraction developed a wine-color with the phenol sulfuric acid method. The purified α -CD-COOH (Na salt) had R_{Glc} 0.37 and gave IR absorption bands at 1760 (shoulder) and 1620 cm⁻¹ (Fig. 4a), which are characteristic of the carboxyl group. Because the sample was the Na salt of α -CD-COOH, these peaks slightly deviated from the standard positions of the carboxyl group. Moreover, ¹³C-NMR spectra of α -CD-COOH (Na salt) also gave evidence for the presence of a carboxyl group (three new resonances at 179.8~181.5 ppm) in addition to the signals of α -CD (Fig. 4b). Although these results would not be sufficient for the quantitative evaluation of the dialdehyde structure of dial-CD, we judged that the periodate oxidation of CD was useful to introduce a dialdehyde structure at the C-2 and C-3 positions of the D-glucose residue in the CD molecule.

Isomers of dial-CD I

The stoichiometric consumption of periodate with time (Fig. 1) was not compatible with the results of gel filtration of the product on the Sephadex G-15 column (Fig. 3), where

about 67% of the α -CD was shown to be unoxidized. Moreover, ^{13}C -NMR analysis of the dial- α -CD I and its dicarboxylate gave complex chemical shift patterns, which would be attributable to the occurrence of various

SCHEME. Possible Isomers of the Dial- α -CD Produced by the Periodate Oxidation of α -CD.

I ~ VI represent the numbers of D-glucose residues oxidized into the dialdehyde structure. A ~ F represent the positions of dialdehyde-glucose in the CD molecule.

TABLE I. MALTOOLIGOSACCHARIDES DERIVED FROM THE DIAL-CD ISOMER FORMS BY NaBH_4 AND ACID TREATMENTS^a

Dial-CD isomer	Theoretical value (mol) ^b					Experimental value (mol)				
	M ₅	M ₄	M ₃	M ₂	G	M ₅	M ₄	M ₃	M ₂	G
IA	1					+				
IIAB		1			1		+			+
IIAC			1					+		
IIAD				2					+	
IIIABC			1					+		
IIIABD			1	2				+	+	
IIIACD				1	1				+	+
IIIACE					3					+
IVABCD				1					+	
IVABDE					2					+
IVACDE					2					+
VABCDE					1					+
VIABCDEF										
Total	1	1	3	6	10	1	4	14	7	8

^a M₅ ~ M₂ and G represent maltopentaose-maltose and glucose, respectively.

^b Theoretical values were calculated based on the case in which all isomers were produced in equal amounts (1 mol).

forms of oxidized α -CD. Therefore, the route A shown in the Scheme may not be possible, and several forms of the possible isomers would be produced by the oxidation (Scheme, route B). The isomers were grouped with the numbers (I~VI) and positions (A~F) of the D-glucose molecules oxidized into dialdehydes (Scheme). Separation of these isomers by paper chromatography was not attained as described above.

Thus, the composition of the isomers was analyzed by the measurement of maltooligosaccharides produced by the NaBH_4 reduction of dial- α -CD I, subsequent acid cleavage of alcohol groups, and separation by paper chromatography. Table I shows the composition of maltooligosaccharides derived from the respective isomer forms. Maltopentaose and maltotetraose theoretically originated from isomers IA and IIAB, respectively.

Maltotriose was derived from the isomers IIAC and IIIABC. Maltose and glucose originated from several forms of the isomers. When we suppose all isomers are produced in equal amounts, the theoretical value of the ratio of maltopentaose to glucose should be 1:1:3:6:10. In contrast, the experimental value of 1:4:14:7:8 (Table I) was obtained, which showed the amounts of maltotetraose and maltotriose were obviously higher than those of the supposed values. Therefore, the dial-CD I has an average of 3.5 glucose residues oxidized into the dialdehyde structure.

Reactivity of dial-CD I

Reactions between the dial-CD I and amino acids were examined to confirm the reactivity of the dialdehyde group. As shown in Fig. 6a, 30 mM lysine incubated with the crude dial-CD I (30 mM) at 30°C for 3 hr in the dark was eluted from the Sephadex G-15 column at a position much faster than that of control lysine. Although reduction of the reaction mixture with NaBH_4 decreased the intensity of carbohydrate (A_{490} value by the phenol sulfuric-acid method), the eluting position of the lysine-dial-CD adduct was not changed (Fig. 6c). With glycine, the amounts of dial-

FIG. 6. Gel Filtration Analysis of the Amino Acid- α -CD I Adducts on a Sephadex G-15 Column.

Periodate-oxidized α -CD I (30 mM, the mixture of dial-CD and native CD) was mixed with lysine or glycine, 30 mM each, and 0.1 M triethanolamine buffer, pH 8.5 (total 200 μ l), and incubated at 30°C for 3 hr in the dark. In separate experiments, the reaction mixture was reduced with 30 mM NaBH_4 and then acidified and neutralized. The reaction mixture was put on a column (1.5 $\times$ 8.0 cm) of Sephadex G-15 and eluted with water. Fractions (0.17 ml (4 drops)/tube) were collected and assayed for total sugar (A_{490} , ----) and amino acid (fluorescence, —). (a) lysine-dial-CD adduct, (b) glycine-dial-CD adduct, (c) reduced form of lysine-dial-CD adduct, (d) reduced form of glycine-dial-CD adduct. Arrows in (a) and (b) show the eluting positions of authentic lysine and glycine, respectively.

CD adduct were significantly smaller than with lysine (Fig. 6b). However, the NaBH_4 reduction changed the eluting position of the adduct to somewhat later than that of the control glycine. Comparison of Figs. 6a and b showed that lysine was more reactive than glycine, yielding larger amounts of adduct. Difference between the elution patterns of lysine- and glycine-dial-CD adducts after the reduction should be examined in detail.

DISCUSSION

Several analyses of the periodate-oxidized

product of native CD, *i.e.*, measurement of consumption of periodate, production of reducing power, production of formic acid, and the numbers of residual intact D-glucose molecules (see RESULTS), confirmed the cleavage of the linkage between C-2 and C-3 of the cyclic α -1,4-linked D-glucose residues, and the introduction of dialdehyde groups in the α -CD molecule. Overoxidation to formic acid and formaldehyde was negligible. However, the IR spectra of dial- α -CD I gave no clear evidence for the occurrence of a dialdehyde structure. This might be because of the formation of hydrated forms, that is hemiacetal and hemialdal forms of the dialdehyde.^{15,16)}

Therefore, dial- α -CD I was further converted into the dicarboxylated form by hypiodite oxidation.⁹⁾ The α -CD-COOH was purified by a simple filtration on a Bio-Gel P-2 column, where the carboxyl-derivative was eluted at the void volume of the column. The separation of negatively charged compound from the neutral compound could be explained by the occurrence of a weakly charged structure of the Bio-Gel. Similar separation was reported for the phosphate derivative of manno-oligosaccharide¹⁷⁾ and the oligosaccharides containing uronic acid.¹⁸⁾

Evidence for the carboxyl group in the α -CD-COOH was provided by IR and ¹³C-NMR spectra (Fig. 4). These analyses gave a strong support to the idea of dialdehyde structure of periodate-oxidized CD.

Since the dial-CD has possible 13 isomer forms, the composition of the periodate-oxidized product of dial-CD I was examined after conversion into maltooligosaccharides (Table I). Based on the ratio of fragment oligosaccharides, on the average 3.5 glucose residues in the CD molecule contained dialdehyde groups, and this led to the value of 4.85 for the average numbers of glucose residues unoxidized. By assuming that about 67% of CD remained unoxidized (Table I), this value showed a good correspondence to the value (4.68) obtained by the direct estimation of numbers of glucose residues unoxidized. It is interesting to note that more than half of the

CD remained unoxidized in spite of the almost complete consumption of sodium metaperiodate (Fig. 1). Increase in the ratio of NaIO₄ to the α -CD molecule would lead to the increase in the proportion of highly oxidized isomers such as IV, V, and VI shown in the Scheme. Like the adenosine-dialdehyde,¹⁹⁾ the dial-CD I readily reacted upon incubation at 30°C for 3 hr in the dark, with the NH₂-groups of glycine and lysine with or without NaBH₄ reduction (Fig. 6). The reactions between the aldehyde group of carbohydrates and NH₂-group of amino acids or peptides were extensively studied as a Maillard reaction.^{20,21)} In the case of affinity labeling with oxidized ATP, dialdehyde groups were supposed to react with the ϵ -NH₂ group of lysine by Schiff base formation.²²⁾ The highly reactive property of the dialdehyde group in the dial- α -CD I and dial- α -CD II was verified by the enzymatic experiments shown in the following paper.

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