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# Action of Taka-amylase A and cyclodextrin glucanotransferase on dialdehyde-cyclodextrins and their reduced forms

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## ABSTRACT

Cyclomalto-oligosaccharides ($\alpha$-, $\beta$-, and $\gamma$-cyclodextrins) were partially oxidized by sodium meta-periodate, yielding the dialdehyde forms (D-CDs). Reduction of the D-CDs by sodium borohydride gave the corresponding alcohols (D-CD/Rs). Column chromatography on Sephadex G-15 was effective in separating cyclomalto-hexaose and -heptaose ($\alpha$- and $\beta$-CD) derivatives from native CD. The susceptibility of $\alpha$- and $\beta$-CD to Taka-amylase A increased markedly on conversion into derivatives, as judged by the decrease in the $K_m$ values. D-CDs were better substrates for cyclodextrin glucanotransferase than the D-CD/Rs and native CDs when the reaction assayed was hydrolysis of the CD. The observed increases in susceptibility to enzymes could be ascribed to a loosening of the cyclic structure of the substrates by the oxidative cleavage of carbon–carbon bonds.

## INTRODUCTION

Cyclomalto-oligosaccharides (cyclodextrins, CDs) are known to have various unique characteristics and practical uses[1]. Moreover, many CD derivatives have been synthesized with a view to the chemical construction of enzyme mimics[2]. In our series of studies, CD dialdehydes (D-CDs) were prepared by periodate oxidation[3], and the actions of the D-CDs with amylases and phosphorylases were shown to be inhibitory at neutral pH and cause irreversible inactivation at alkaline pH[4–6].

The periodate-oxidized dialdehyde derivatives of ribonucleotides have been shown to react with lysine residues of proteins to form Schiff base adducts[7,8]. Similarly, basic studies by Schwartz and Gray[9] have clarified the reaction between reducing disaccharides, such as maltose and lactose, and bovine serum albumin. In these studies, the high reactivity of the aldehyde group of sugars with the $\epsilon$-amino groups of lysine residues of proteins was clearly demonstrated.

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Various substrate analogs and derivatives have been used to examine the specificity of carbohydrases, and these studies have provided useful information on catalytic mechanisms, as shown[10] in the case of Taka-amylase A. Other interesting findings with substrate derivatives are illustrated by the work of Skold et al.[11], who showed that the conversion of *o*-nitrophenyl β-galactoside into high molecular weight forms promoted the enzymic hydrolysis of the compound and decreased its $K_m$ value. Therefore, the modification of a substrate molecule may enhance enzyme action. We examined the action of various enzymes related to amylase on D-CDs and their derivatives to ascertain the effects of dialdehyde groups. Because D-CD worked as an irreversible inactivating reagent of amylase at alkaline pH[4], the present experiments were done at pH 5.2.

## RESULTS

*CD-dialdehydes and derivatives.*—The α-, β-, and γ-cyclodextrins (α-, β-, and γ-CDs) (60 mM) were oxidized with equimolar sodium metaperiodate at 25°C in the dark. The initial velocities of oxidation of the three CDs were in the decreasing order γ, β, α. Methyl α-D-glucopyranoside gave an oxidation curve similar to that of β-CD, whereas the water-insoluble methyl 4,6-*O*-benzylidene-α-D-glucopyranoside was oxidized very slowly (Fig. 1a). An increase in reducing power indicated the progress of oxidation and evidenced the production of dialdehyde groups (Fig. 1b).

As shown previously[3], a Sephadex G-15 column was effective in separating the α-CD-dialdehyde (D-α-CD) from unoxidized cyclomaltohexaose (Fig. 2a). Though the β-CD-dialdehyde (D-β-CD) was also separated from unoxidized, native cyclomaltoheptaose (Fig. 2b), the reaction mixture from the oxidation of cyclomalto-octaose gave a single peak of total sugar, which corresponded with the peak of the reducing substance (Fig. 2c). When the oxidized reaction mixtures were reduced with sodium borohydride and subjected to the Sephadex G-15 column, the reduced

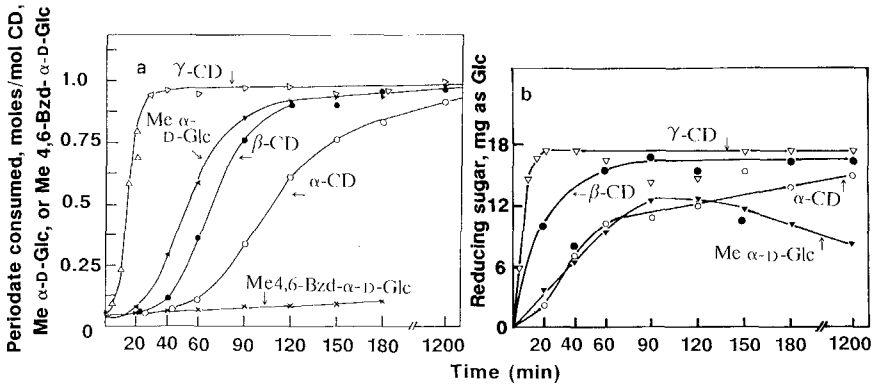


Fig. 1. Periodate oxidation of CDs and glucose derivatives.

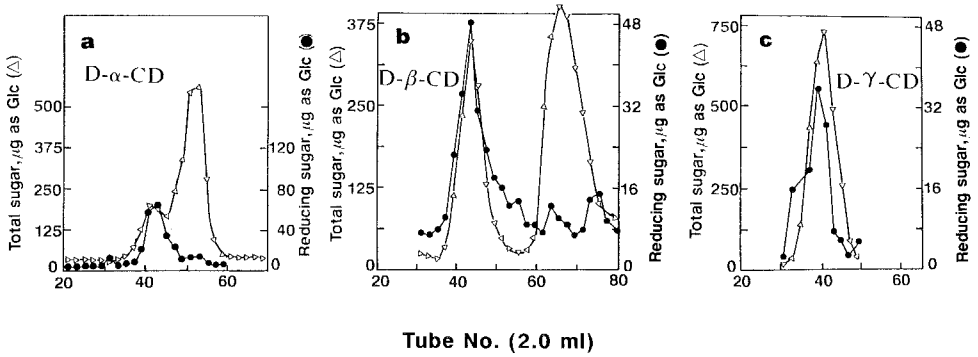


Fig. 2. Elution patterns of D-CDs from a Sephadex G-15 column. The reaction mixture ( ~ 20 mg in 500 μL) was injected onto the column, and total sugar ( ▽ ) and reducing power (●) were monitored during elution with water.

forms of D-α-CD and D-β-CD (D-α-CD/R and D-β-CD/R) were similarly separated from the starting CD, more than 50% of which remained unoxidized.

The dicarboxyl derivative of α-CD (α-CD-COOH), prepared by the hypoiodite oxidation[12] of D-α-CD, was eluted at the void volume of a Bio-Gel P-2 column[3]. Similarly the dicarboxyl derivative of γ-CD (γ-CD-COOH) was purified as the void volume fraction from a Bio-Gel P-2 column (data not shown). As mentioned previously[13], other negatively charged CD derivatives, such as D-α-CD–amino-caproic acid complex, are also eluted at the void volume of a Bio-Gel P-2 column.

*Action of Taka-amylase A.*—Since extensive studies have been made on Taka-amylase A (TAA), which is a representative fungal α-amylase isolated from the culture medium of *Aspergillus oryzae*, various kinetic data are available to characterize the action pattern[14]. Therefore, a comparison of the susceptibility of the three D-CDs and their derivatives was made with this enzyme. Although the difference between the native α-, β-, and γ-CDs is a span of only 1 or 2 glucosyl residues out of 6–8 residues, a significant difference in the susceptibility of the CDs to hydrolysis by TAA was observed (Fig. 3a). The α-CD was hydrolyzed slowly and to a small maximal extent after 40 min; only 0.03 mol glucose equivalent/mol CD was formed. In contrast, β- and γ-CD were hydrolyzed rapidly and showed a much higher maximal extent of hydrolysis (0.33 mol glucose equivalent/mol CD) under the same conditions.

A pronounced increase in the susceptibility of D-α-CD was attained by periodate oxidation. The susceptibility of D-β-CD and especially of D-γ-CD was greater than that of the D-α-CD; however, the observed final extent of hydrolysis of D-β-CD and D-γ-CD was only about one-third or one-half that of the respective native CDs (Fig. 3b). The data in Fig. 3c show the limits of hydrolysis of D-α-CD/R and D-β-CD/R to be closer to that of D-γ-CD/R than was found for the native and D-CDs. However, the reason appears to be the much reduced hydrolysis limit of the D-γ-CD/R compared to D-γ-CD and γ-CD.

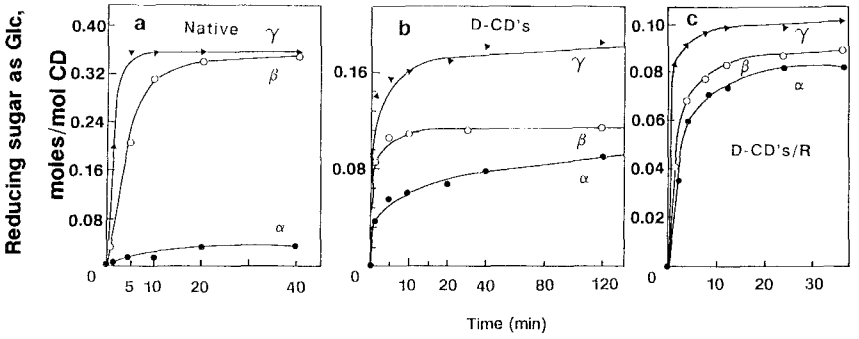


Fig. 3. Hydrolysis of CDs and their derivatives by Taka-amylase A.

To obtain more quantitative information on TAA action, kinetic constants for the CDs and their derivatives were evaluated (Table I). Values for the native CDs were comparable with the reported values[15], shown in the parentheses, and judging from the $K_m$ and $V_{max}$ values γ-CD was the best substrate for TAA among the three CD forms. Based on the apparent secondary velocity constant, $V_{max}/K_m$, the derivatives of α- and β-CD were better substrates than the respective native CDs, but D-γ-CD and D-γ-CD/R were slightly poorer substrates than γ-CD.

*Action of CGTase.*—Cyclomaltodextrin glucanotransferase (CGTase, EC 2.4.1.19) is known to produce three CDs from the starch molecule. Besides the intrinsic reactions of CGTase (i.e., cyclization, coupling, and disproportionation) this enzyme catalyzes the hydrolysis of the CD molecule itself[16]. Therefore, the efficiency of the CDs and their derivatives as hydrolyzable substrates for CGTase from *Bacillus macerans* was examined. As revealed in Fig. 4, α-CD showed an almost linear progress in the increase in reducing power. D-α-CD gave a concave-

TABLE I

Kinetic constants for Taka-amylase A action on cyclodextrins and their derivatives [a]

| Cyclo-dextrin | Native | | Dialdehyde | | Reduced dialdehyde | |
|---|---|---|---|---|---|---|
| | $K_m$ (mM) | $V_{max}$ [b] | $K_m$ (mM) | $V_{max}$ [b] | $K_m$ (mM) | $V_{max}$ [b] |
| α-CD | 6.8 (4.7) [c] | 0.8 | 2.3 | 1.2 | 1.9 | 1.1 |
| β-CD | 8.9 (10.2) | 3.7 | 2.1 | 1.9 | 4.1 | 3.1 |
| γ-CD | 4.9 (2.4) | 11.9 | 8.5 | 14.8 | 22.3 | 47.1 |

[a] Reaction mixtures contained Taka-amylase A [6 μg (0.72 units) in 10 μL] and substrate (90 μL, 1.2–20 mM in 40 mM acetate buffer, pH 5.2). After 5 min at 30°C, reaction was stopped by the addition of M NaOH (25 μL). An aliquot (25–100 μL) was subjected to reducing-sugar analysis by the Neocuproine method. [b] Expressed as μmol glucose/min/μg enzyme. [c] Values in parentheses are from ref. 15.

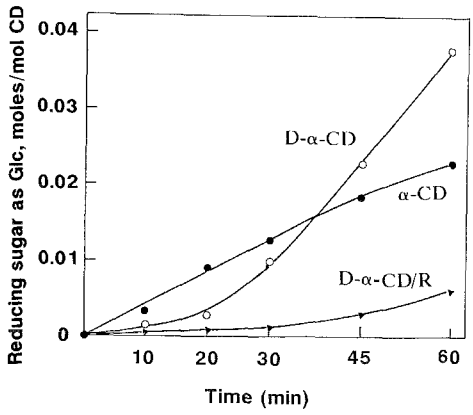


Fig. 4. Hydrolysis of α-CD and its derivatives by cyclodextrin glucanotransferase.

upward curve, reflecting continuous increase in velocity up to 40 min of incubation. In contrast to D-α-CD, D-α-CD/R was a poor substrate for CGTase.

The hydrolysis and coupling reactions of CGTase on CDs and their derivatives were compared (Fig. 5). Although the decrease in Glc values seen in the presence of phenyl α-D-glucoside might a priori indicate an inhibition of the hydrolysis, paper chromatographic analysis showed a large increase in the coupling products with D-γ-CD. The α-, β-, and γ-D-CDs gave much the highest values for both hydrolysis and coupling, and particularly D-γ-CD was the best substrate among the six forms including native CDs. For the reduced forms of D-CDs (D-CD/Rs), rather effective coupling reactions were observed for the three forms.

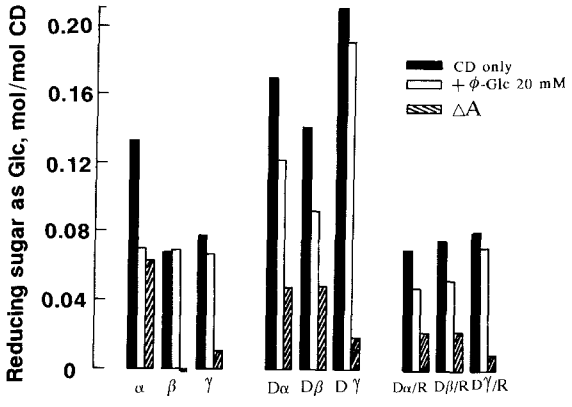


Fig. 5. Action of cyclodextrin glucanotransferase on CDs and their derivatives. Hydrolysis of CD (1.5 mM) and derivatives was measured by the increase in reducing power after incubation at 30°C overnight (■). For the coupling reaction mixtures contained 20 mM phenyl α-D-glucoside in addition to CD (1.5 mM) and enzyme. The overnight increase in reducing power (□) was diminished owing to the transfer of some CD fragments to the acceptor, phenyl α-D-glucoside. The difference between the above two values (▨) represents the efficiency of the CD in the transfer reaction.

*Analysis of the reaction products.*—When comparing the action of TAA and CGTase on CD derivatives, it is important to check whether the cyclic structure is retained in the derivative forms or not. Thus, the action of glucoamylase on D-β-CD was carefully examined. After an overnight incubation the reaction products were analyzed by paper chromatography (data not shown). In addition to the spot for D-β-CD ($R_G$ 0.43), a distinct spot corresponding to standard glucose was observed. However, quantitative measurement showed that the amount of glucose formed was less than 10% of the total sugar. By contrast, the action of TAA on D-β-CD gave strong spots for glucose and maltose. Several spots having $R_G$ values higher than that of glucose might have represented oligosaccharides having a one ring-opened residue in their structures. The main products from the action of CGTase on β-CD, D-β-CD, and D-β-CD/R, were glucose and maltose (data not shown).

## DISCUSSION

Cyclodextrins have neither reducing nor nonreducing terminals because of their cyclic structure. The interior of the ring is hydrophobic and the exterior hydrophilic. As shown in Fig. 2, on treatment with periodate more than half the α- and β-CD remained unoxidized, even though equimolar concentrations of reagent and CD were used in the reaction. This could be explained by the formation of a complex between the dialdehyde groups of oxidized CD molecules and the hydrophobic or hydrophilic moiety of intact CD molecules[17]. Moreover, separation of the D-CD and D-CD/R derivatives from the intact α- and β-CDs by the Sephadex G-15 column might be attained not by the gel filtration principle but by changes in affinity for the G-15 gel caused by the oxidation and reduction.

The poor susceptibility of α-CD to TAA was greatly improved by conversion into the D-CD and D-CD/R forms. D-β-CD and D-β-CD/R are also more susceptible to TAA than β-CD. These changes are represented by increases in the initial velocities (Fig. 3) and decreases in the $K_m$ values (Table I). However, the γ-CD derivatives became less susceptible, although their $V_{max}$ values increased. The order of susceptibility of CDs to TAA corresponded to their susceptibility to periodate oxidation, which might be explained by the size of the cavity of the CD molecules. Thus the increase in the susceptibility of a CD derivative to TAA hydrolysis could be ascribed to a loosening of the cyclic structure resulting from the cleavage of carbon bonds by periodate.

The rate of hydrolysis of CDs by CGTase was in the increasing order α, β, γ (Fig. 5). Hydrolysis of D-α-CD and D-α-CD/R gave concave-upward curves, with the former showing more susceptibility than α-CD after about 40 min of reaction (Fig. 4). The later, more rapid formation of reducing sugars in the D-α-CD digest would suggest that the linear "dialdehyde" saccharide produced from D-α-CD is hydrolyzed faster than α-CD or D-α-CD. Upon overnight incubation all D-CD forms showed a higher reducing power than the native CDs (Fig. 5). In the

coupling reaction with phenyl α-D-glucoside, the glucoside appeared to compete with water as an acceptor most effectively when the substrates were α-CD, D-α-CD, and D-β-CD.

Analysis of the hydrolysis product from D-β-CD by glucoamylase reaction gave glucose equal to less than 10% of the total sugar. Because CD molecules, in contrast to linear malto-oligosaccharides, are not susceptible to glucoamylase, the unreactivity of the D-CD molecules suggests that they retain their cyclic structure. The TAA digestion of D-β-CD gave glucose and maltose as reaction products, showing that maltose units persisted in the D-CD form. Although periodate sufficient to oxidize only one glucose unit in each CD molecule was supplied (and consumed, Fig. 1a), the D-α-CD and D-β-CD isolated by chromatography (Fig. 2a,b) were accompanied by more than an equal amount of unreacted CD.

## EXPERIMENTAL

*Reagents.*—Crystallized preparations of Taka-amylase A (TAA) and lyophilized, amorphous glucoamylase were purchased from Seikagaku Kogyo Co. Cyclodextrin glucanotransferase (CGTase from *Bacillus macerans*) was the product of Amano Pharmaceutical Co. Polyacrylamide gel electrophoresis and subsequent measurements of activity eluted from slices of the gel showed that this enzyme preparation gave a single, major band of α-CD-hydrolyzing activity, which corresponded to the starch-hydrolyzing activity. The α-, β, and γ-CDs were the products of Nihon Shokuhin Kako Co. Other reagents were of analytical grade.

*Periodate oxidation.*—CDs, methyl α-D-glucoside, and methyl 4,6-*O*-benzylidene-α-D-glucoside (30 mmol each) were oxidized with $NaIO_4$ (30 mmol) at 25°C for 20 h in the dark. Samples (25 μl) were removed at intervals, diluted 40-fold with water, and the UV absorbance at 222.5 nm was measured (consumption of $NaIO_4$[18]) and reducing power (Nelson–Somogyi method[19]). In work on the preparative scale, excess $NaIO_4$ was destroyed with ethylene glycol.

*Preparation of CD derivatives.*—CD-dialdehydes (D-CDs) were reduced by $NaBH_4$ at 25°C in the dark, and excess $NaBH_4$ was removed by HCl treatment and evaporation. These reduced forms of D-CD (D-CD/Rs) were further purified by gel filtration on a Sephadex G-15 column.

*TAA and glucoamylase digestion.*—CDs and their derivatives were hydrolyzed as follows: Taka-amylase A solution [0.6 mg (72 units) per mL in 40 mM acetate buffer, pH 5.2] was mixed with an equal volume of substrate solution (5 mg/mL) and incubated at 30°C. The reaction was stopped by the addition of M NaOH and the reducing sugar was assayed by the Neocuproine method[20] ($A_{450nm}$) as shown in Fig. 3. Glucoamylase digestion was done using 1 mg enzyme per mL of 40 mM acetate buffer, pH 5.2 (31.5 U/mg).

*CGTase digestion.*—CDs and their derivatives were hydrolyzed by essentially the procedure just described. The CGTase solution contained 1 mg enzyme (3.6 units) per mL of 40 mM acetate buffer, pH 5.2.

*Paper chromatography.*—The enzymic hydrolyzates were analyzed by paper chromatography using aq 65% *n*-propanol as a solvent. Spots were revealed by the silver nitrate-dip procedure. For quantitative measurement, chromatograms were cut into 5-mm strips, these were eluted with water, and total sugar in each eluate was determined by the phenol–$H_2SO_4$ method.

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