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Some physicochemical properties of branched β-cyclodextrins and their inclusion characteristics

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Summary

The physicochemical and biological properties, chemical and enzymatic stabilities and inclusion abilities of branched β-cyclodextrins, such as glucosyl-β-cyclodextrin (G_1-β-CyD), maltosyl-β-CyD (G_2-β-CyD) and di-maltosyl-β-CyD ($(G_2)_2$-β-CyD), were studied, comparing with those of parent β-CyD. Branched β-CyDs had much higher aqueous solubility (> 50%) than the parent β-CyD, but no surface activity was observed. The hemolytic activity of branched β-CyDs was weaker than that of parent β-CyD and decreased with increasing number of branched glucose units, which was well correlated with their ability to remove cholesterol from human erythrocytes. The acid-catalyzed degradation rate increased in the order of: β-CyD ≈ G_1-β-CyD < G_2-β-CyD < $(G_2)_2$-β-CyD. The three β-CyDs were resistant to the α-amylase-catalyzed hydrolysis, whereas G_2- and $(G_2)_2$-β-CyDs were hydrolyzed by pullulanase and glucoamylase at appreciable rates. Inclusion ability of branched β-CyDs generally decreased with increasing number of branched glucose unit, which may be due to the steric hindrance of glucose or maltose residues.

Introduction

Branched cyclodextrins, in which primary hydroxyl group of cyclodextrins is substituted by mono- and disaccharides through the α-1,6 glycosidic linkage, have recently received considerable attention in pharmaceutical field because of their high aqueous solubility (Abe et al., 1986; Koizumi et al., 1986; Koizumi et al., 1987). In this study, some physicochemical properties of 6-O-α-D-glucosyl-β-CyD (G_1-β-CyD), 6-O-α-D-maltosyl-β-CyD (G_2-β-CyD) and $6^A,6^D$-di-O-α-maltosyl-β-CyD ($(G_2)_2$-β-CyD), such as surface and hemolytic activity, were investigated. Furthermore, chemical stabilities of the branched β-CyDs against acid and enzymes such as α-amylase, glucoamylase and pullulanase were studied and compared with those of parent β-CyD. In addition, the solubilization effects of branched β-CyDs against several poorly water-soluble drugs were surveyed to evaluate their inclusion ability.

Materials and Methods

Materials

β-CyD was supplied by Nippon Shokuhin Kako Co. (Tokyo, Japan). G_1-, G_2- and $(G_2)_2$-β-CyDs

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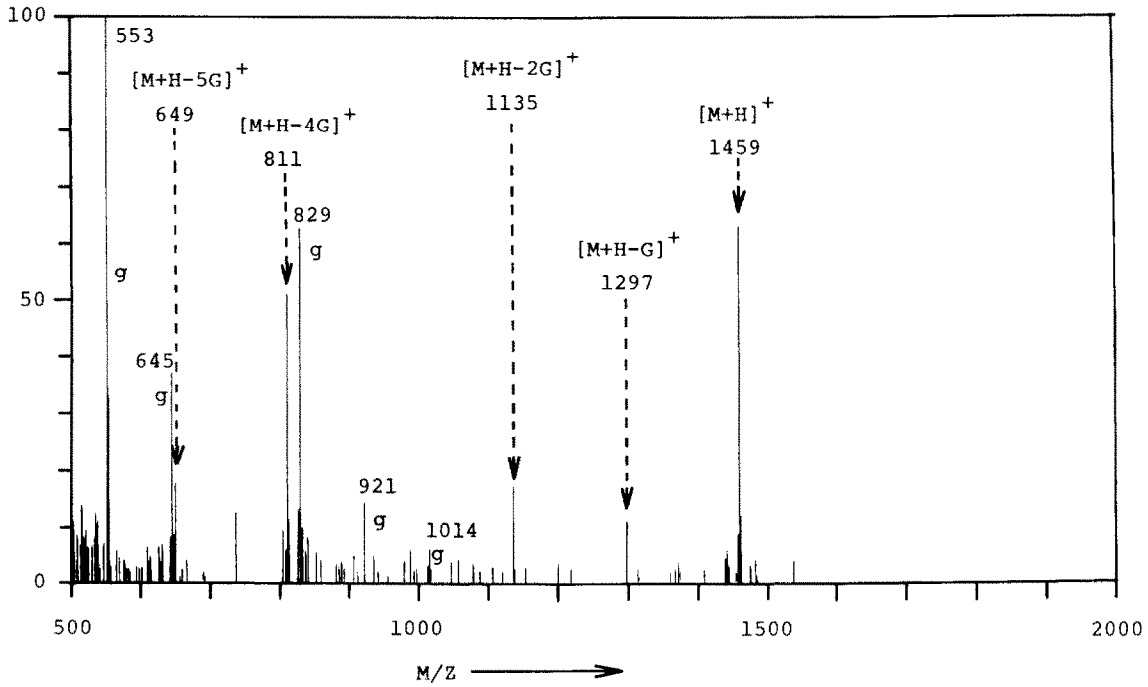


Fig. 1. Mass spectrum of G_2-β-CyD. G, glucose unit; g, glycerol.

were donated from Tokuyama Soda Co. (Tokuyama, Japan), and characterized by secondary ionization mass spectroscopy (SIMS). In mass spectra of G_1-, G_2- and $(G_2)_2$-β-CyDs, they gave high parent-ion peaks at 1297, 1459 and 1783, respectively, corresponding to the molecular weights plus proton $(M + H)^+$, from which glucose unit was split stepwise. A mass spectrum of G_2-β-CyD, as an example, is shown in Fig. 1. α-Amylase (*Aspergillus oryzae*), glucoamylase (*Rhizopus niveus*) and pullulanase (*Aerobacter aerogenes*) were purchased from Seikagaku Kogyo (Tokyo, Japan). Other chemicals and drugs were from commercial sources, and de-ionized double distilled water was used.

Apparatus

Optical rotation measurements: DPI-360 digital polarimeter (Jasco, Tokyo, Japan) with an accuracy of ±0.002°. Surface tension measurements: duNouy surface tensionmeter (Shimadzu Co., Kyoto, Japan) with an accuracy of $\pm 0.5\ mN \cdot m^{-1}$. SIMS spectra: double focusing M-80 B mass spectrometer (Hitachi, Tokyo, Japan) with xenon gas as a primary ion source and glycerol as the matrix; the primary and secondary ion acceleration were 8 and 3 kV, respectively.

Hemolysis studies

Human erythrocytes from freshly drawn blood were supplied by the Kumamoto Prefectural Red Cross Blood Center, Japan. Erythrocytes were separated by centrifugation ($1000 \times g$ for 10 min), washed 3 times with isotonic phosphate buffer (pH 7.4) and resuspended to give a hematocrit of 5%. Aliquots (0.1 ml) of the erythrocyte suspension were added to CyD solutions (4 ml) and the mixture was gently agitated for 30 min at 37°C. After centrifugation ($1000 \times g$ for 10 min), the optical density of the supernatant was measured for hemoglobin at 543 nm. Results were expressed as % total hemolysis by comparison with a sample of the complete hemolysis in water. Concentrations of CyDs to induce the 50% hemolysis were calculated according to Reed and Muench (Ishii et al., 1983).

Release of cholesterol from human erythrocytes

The erythrocyte suspension was treated with β-CyDs under the similar condition to that of hemolysis studies described above, except for β-CyD concentrations. The concentration of β-CyDs was below 3 mM where no hemolysis occurred. Cholesterol in the supernatant (3 ml) of erythrocyte suspension was extracted with ether (6 ml) containing cholesterol acetate as an internal standard for gas chromatography. 5 ml of the organic phase was evaporated and the residue was dissolved in 100 μl of dichloromethane, 3 μl of which was subjected to gas chromatography for determination of cholesterol. The chromatograph (Shimadzu GC-6A, Kyoto, Japan) was operated using N_2 as a carrier gas at a flow rate of 40 min/ml. The column was of coiled column (3 mm diameter × 500 mm) packed with 3% OV-17 on 80–100 mesh Chromosorb WHP (Tokyo, Japan). The temperatures of the injection and column ports were 280 and 255 °C, respectively.

Kinetics

Acid-catalyzed hydrolysis: hydrolysis of β-CyDs (2.5×10^{-3} M) was carried out at 60 °C in 0.1 M HCl–KCl solution ($\mu = 0.2$). A 0.5 ml portion of reaction solution was taken at appropriate intervals and neutralized by adding 0.1 N NaOH containing α-CyD as an internal standard for high-performance liquid chromatography (HPLC), 20 μl of which was subjected to HPLC for simultaneous determination of $(G_2)_2$-β-CyD, G_2-β-CyD, G_1-β-CyD and β-CyD (see Fig. 2). The HPLC condition was as follows: pump, Hitachi 655A (Tokyo, Japan); detector, Shodex SE-31 differential refractometer (Showa Denko, Tokyo, Japan); column, ERC-NH-1181 (6 mm diameter × 200 mm) (Erma Optical Works, Tokyo, Japan); mobile phase, acetonitrile–water (65 : 35 v/v); flow rate, 1.2 ml/min. The hydrolysis at pH 1.0 was followed for 3 or more half-lives, while those at pH 1.5 and 2.0 were for about a half-life because of the slow rate. The kinetic data conformed to the first-order rate law. The pH of the sample solution was ascertained to be identical before and after the reaction.

α-Amylase-catalyzed hydrolysis: the enzyme reaction was carried out in 0.05 M acetate buffer (pH 5.2) containing 5 mM $CaCl_2$ at 37 °C. The concentrations of the substrates and α-amylase were 1 ~ 20 mM and 2 units/ml, respectively. At appropriate intervals, the reaction was stopped by boiling the sample solution in water bath. After addition of α-CyD as an internal standard, the remaining CyD was determined by HPLC according to the method described above.

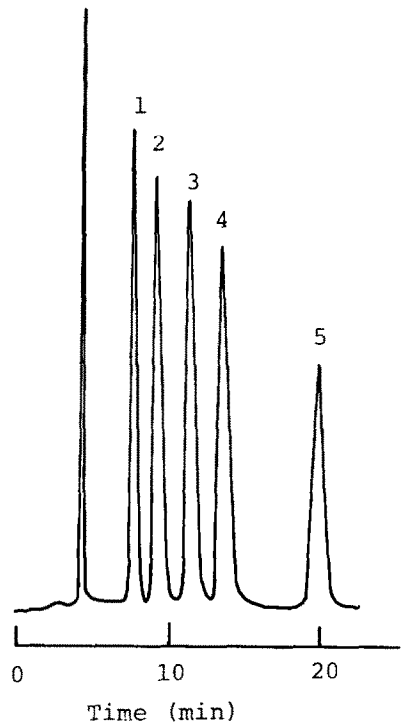


Fig. 2. Liquid chromatogram of branched β-CyDs. 1, α-CyD (internal standard); 2, β-CyD; 3, G_1-β-CyD; 4, G_2-β-CyD; 5, $(G_2)_2$-β-CyD.

Glucoamylase- and pullulanase-catalyzed hydrolyses: the enzyme reaction was carried out in 0.05 M acetate buffer (pH 5.0) at 37 °C. The concentrations of the substrates, glucoamylase and pullulanase were 1 ~ 20 mM, 0.2 and 2.0 units/ml, respectively. Other procedures were the same as those for α-amylase-catalyzed hydrolysis.

Solubility studies

Solubility measurements were carried out according to Higuchi and Connors (1965). Excess amounts of drugs were added to aqueous solutions containing β-CyDs and shaken at 25 °C. After

equilibrium was attained (about 10 days), filtered aliquots were analyzed by spectrophotometry at suitable wavelengths. Apparent 1 : 1 stability constants (K_c) were calculated from the slope of the straight line portion of the phase solubility diagrams.

Results and Discussion

Physico- and biochemical properties

Table 1 lists some physicochemical and biological properties of branched β-CyDs such as aqueous solubility and optical, surface and hemolytic activities. Branched β-CyDs had much higher aqueous solubility (> 50%) than the parent β-CyD. Values of surface tension of branched β-CyD were about the same as that of water (71 mN/m), suggesting no surface activity. This is in contrast to the case of alkylated derivatives such as methylated, hydroxyethylated and hydroxypropylated β-CyDs which have slightly higher surface activity (Yoshida et al., 1988). Fig. 3 shows hemolytic effects of branched β-CyDs on human erythrocytes in isotonic phosphate buffer. Hemolytic activity of branched β-CyDs was weaker than that of β-CyD, i.e. the concentration to induce 50% hemolysis was in the order of: $(G_2)_2$-β-CyD > G_2-β-CyD ≈ G_1-β-CyD > β-CyD (Table 1). Although the hemolysis started at almost the same concentration, 2.0×10^{-3} M, the concentration dependency was different. The CyD-induced hemolysis is reported to be due to the membrane disruption elicited by the dissolution and removal of membrane components (Irie et al., 1982). Thus, the membrane disrupting ability of branched β-CyDs was evaluated by comparing release profiles of cholesterol, one of the important membrane components, from human erythrocytes treated with branched β-CyDs. As is apparent from Fig. 4, the ability of branched β-CyDs to remove cholesterol was lower than that of parent β-CyD and decreased with increasing number of branched glucose unit. This order was well correlated with the hemolytic activity of branched β-CyDs.

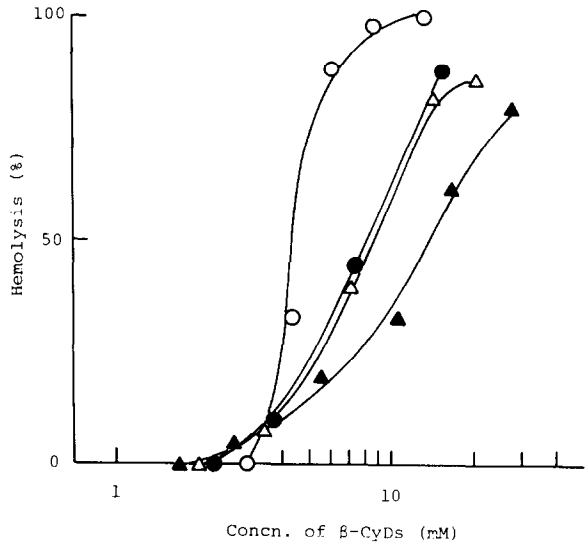


Fig. 3. Hemolytic effects of branched β-CyDs on human erythrocytes in isotonic phosphate buffer (pH 7.4) at 37 °C. ○, β-CyD; ●, G_1-β-CyD; △, G_2-β-CyD; ▲, $(G_2)_2$-β-CyD.

TABLE 1

Some physicochemical properties of β-CyD, G_1-β-CyD, G_2-β-CyD and $(G_2)_2$-β-CyD

CyD	Number of glucose	Molecular weight [a]	Aqueous solubility [b] (g/100 ml)	$(\alpha)_D$ [b]	Surface tension [c] (mN/m)	50% hemolysis (mM)
β-CyD	7	1135	1.85	163	71	5.3
G_1-β-CyD	8	1297	> 50	159	71	8.5
G_2-β-CyD	9	1459	> 50	155	70	9.6
$(G_2)_2$-β-CyD	11	1789	> 50	163	71	14.0

[a] Determined by mass spectrometry (SIMS).
[b] At 25 °C in water.
[c] Concentration of β-CyDs was 0.1% w/v in water.

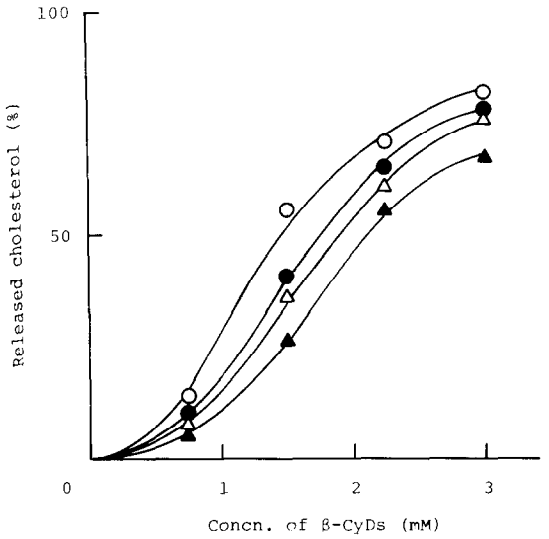


Fig. 4. Release of cholesterol from human erythrocytes treated with branched β-CyDs. ○, β-CyD; ●, G_1-β-CyD; △, G_2-β-CyD; ▲, $(G_2)_2$-β-CyD.

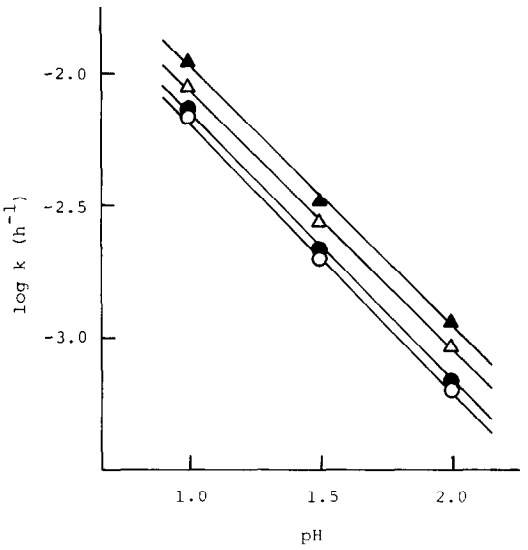
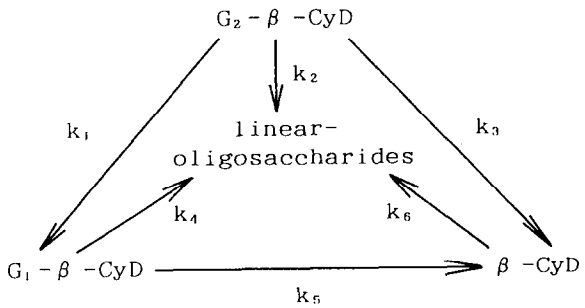


Fig. 5. pH-profiles for acid hydrolysis rate of branched β-CyDs at 60 °C. ○, β-CyD; ●, G_1-β-CyD; △, G_2-β-CyD; ▲, $(G_2)_2$-β-CyD.

Chemical and enzymatic stability of branched β-CyDs

CyDs are known to be fairly stable in alkaline medium, whereas they are hydrolytically cleaved by strong acids to give linear oligosaccharides (Bender and Komiyama, 1978). Fig. 5 shows the log rate constant (k)–pH profiles for degradation of branched β-CyDs in the pH range of 1.0–2.0. The degradation rates showed first-order dependence upon the hydronium ion concentration and increased in the order of: β-CyD ≈ G_1-β-CyD < G_2-β-CyD < $(G_2)_2$-β-CyD. The acid-catalyzed degradation of G_2-β-CyD is thought to proceed according to Scheme 1, where the α-1,4 and α-1,6 glucosidic bonds are hydrolyzed by a complex combination of parallel and consecutive first-order reactions. Thus, each rate constant in Scheme 1 was determined, by monitoring simultaneously the concentrations of G_2-β-CyD, G_1-β-CyD and β-CyD by HPLC (see Materials and Methods) and analyzing the time–conversion profiles by a non-linear least-squares method (Yamaoka et al., 1981). Table 2 lists the hydrolysis rate constants (k_1–k_6) for G_2-β-CyD, together with the partial rate constants for one bond cleavage among seven α-1,4 glucosidic bonds of β-CyD ring, which was calcu-



Scheme 1.

TABLE 2

Rate constants ($\times 10^{-3}$ h^{-1}) for acid hydrolysis of G_2-β-CyD at 60 °C

Rate constant	pH		
	1.0	1.5	2.0
k_1	3.27	0.81	0.26
k_2	5.21 (0.74) [a]	1.79 (0.26)	0.52 (0.074)
k_3	– [b]	–	–
k_4	6.80 (0.97)	1.79 (0.26)	0.55 (0.079)
k_5	–	–	–
k_6 [c]	6.17 (0.88)	1.50 (0.21)	0.44 (0.063)

[a] The values in parenthesis are the partial rate constants.
[b] Could not be determined due to the slow rate.
[c] Determined from the hydrolysis of parent β-CyD.

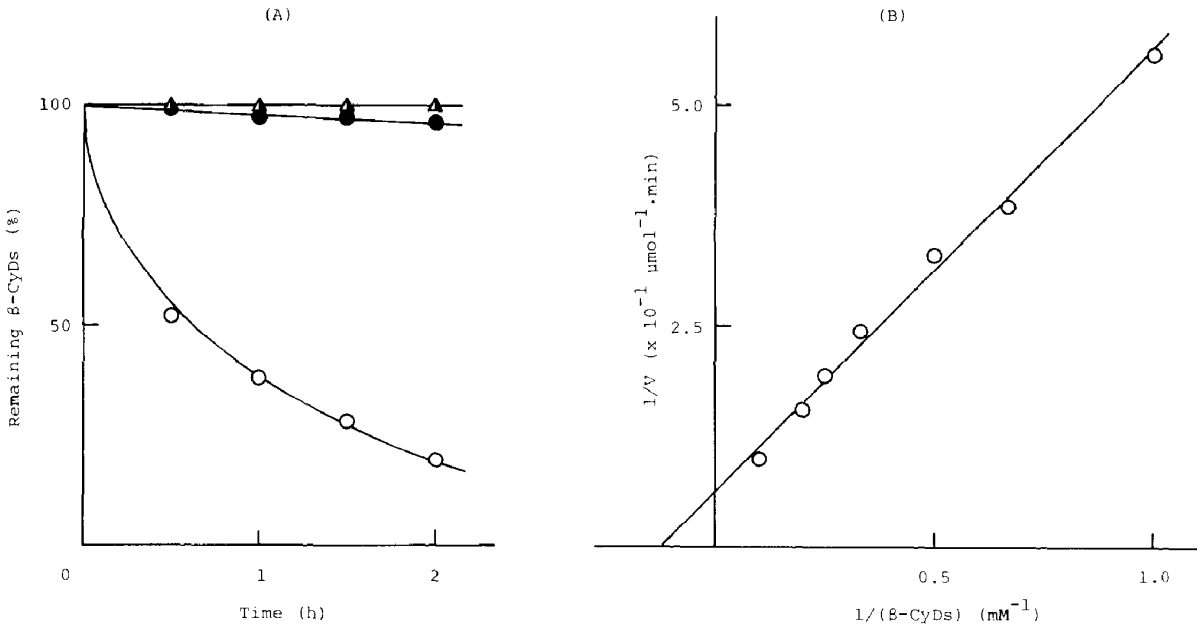


Fig. 6. Time courses (A) for degradation of branched β-CyDs by *Aspergillus oryzae* α-amylase at pH 5.2 and 37°C and its Lineweaver–Burk plot (B). ○, β-CyD; ●, G_1-β-CyD; △, G_2-β-CyD; ▲, $(G_2)_2$-β-CyD.

lated by dividing the overall rate constant by the number of the reactive point. The hydrolysis of α-1,6 glycosidic bond, k_3 and k_5, was negligible under the experimental condition, since β-CyD did not appear in the hydrolyzate of G_1- and G_2-β-CyDs. This is in agreement with the fact that

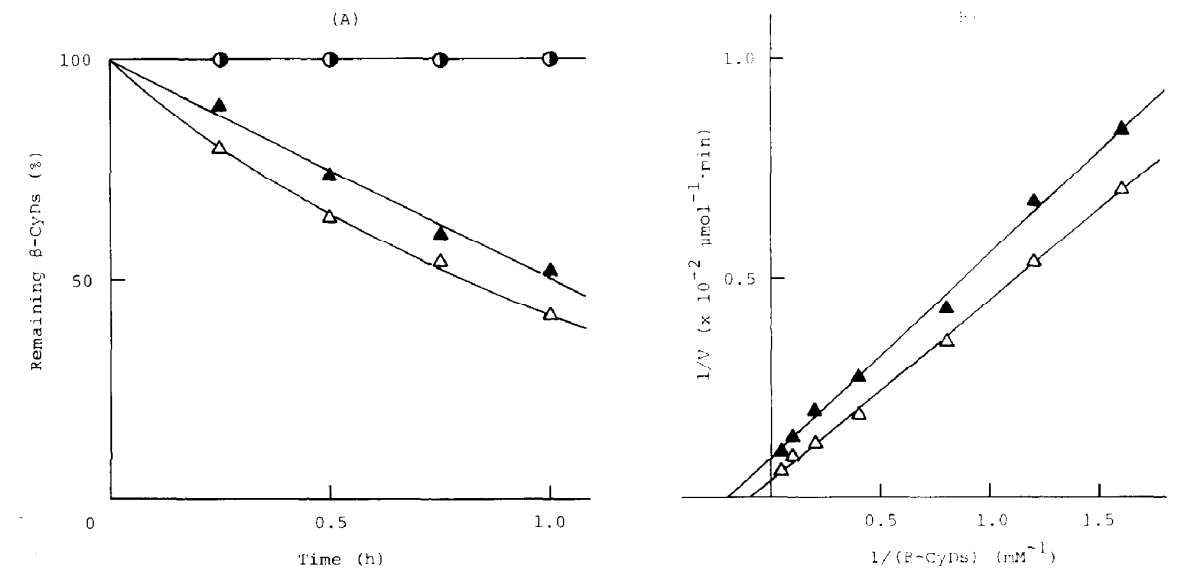


Fig. 7. Time courses (A) for degradation of branched β-CyDs by *Rhizopus niveus* glucoamylase at pH 5.0 and 37°C and their Lineweaver–Burk plots (B). ○, β-CyD; ●, G_1-β-CyD; △, G_2-β-CyD; ▲, $(G_2)_2$-β-CyD.

TABLE 3

K_m and V_{max} values for branched β-CyD-enzyme systems

System	K_m (mM)	V_{max} (μmol/min)
α-Amylase		
β-CyD	8.0	0.16
Glucoamylase		
G_2-β-CyD	7.6	0.20
$(G_2)_2$-β-CyD	5.2	0.11
Pullulanase		
G_2-β-CyD	2.9	0.09
$(G_2)_2$-β-CyD	3.2	0.15

α-1,6 glycosidic bond of oligosaccharides is usually more resistant to acid-hydrolysis than the α-1,4 bond (BeMiller, 1967). It is apparent that the α-1,4 glycosidic bond in the linear maltosyl residue of G_2-β-CyD, k_1, was more susceptible to the hydrolysis than those in the cyclic β-CyD rings, the partial rate constants of k_2, k_4 and k_6, although the overall ring-opening reactions were about 2 times faster than the hydrolysis of the linear maltosyl residue. Furthermore, there was insignificant difference between the ring-opening rates of G_2-β-CyD, G_1-β-CyD and β-CyD.

Fig. 6A shows the time courses of α-amylase-catalyzed hydrolysis of branched β-CyDs at pH 5.2 and 37°C. Parent β-CyD was hydrolyzed by α-amylase at appreciable rate, whereas branched β-CyDs were resistant to the hydrolysis under the experimental condition. Fig. 6B shows the Lineweaver–Burk plot for β-CyD-α-amylase system, from which the Michaelis constant (K_m) and maximal velocity (V_{max}) were determined and listed in Table 3. These values are in good agreement with those reported by Jodál et al. (1984).

On the other hand, G_2- and $(G_2)_2$-β-CyDs were susceptible to glucoamylase that cleaves α-1,4 glycosidic bond from terminal reducing glucose, as shown in Fig. 7A. Of course, β-CyD and G_1-β-CyD were not hydrolyzed by this enzyme because they have no reducing glucose units linked by α-1,4 glycosidic bond. It is apparent from Table 3 that the magnitude of the K_m values was similar for both substrates, whereas the V_{max} of $(G_2)_2$-β-CyD was about half that of G_2-β-CyD in spite of its two reactive points, suggesting the lower molecular activity of $(G_2)_2$-β-CyD against glucoamylase.

Fig. 8A and B show the time courses of the hydrolysis of G_1-, G_2- and $(G_2)_2$-β-CyDs in the presence of pullulanase that cleaves α-1,6 glyco-

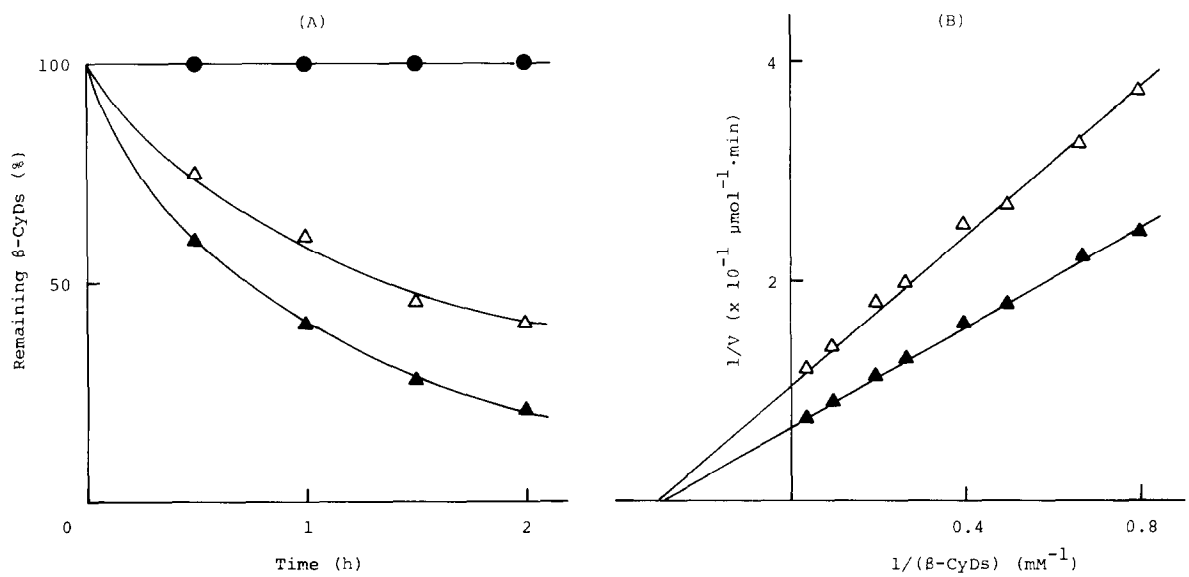


Fig. 8. Time courses (A) for degradation of branched β-CyDs by *Aerobacter aerogenes* pullulanase at pH 5.0 and 37°C and their Lineweaver–Burk plots (B). ●, G_1-β-CyD; △, G_2-β-CyD; ▲, $(G_2)_2$-β-CyD.

TABLE 4

Stability constants (M^{-1}) for inclusion complexes of various drugs with branched β-CyDs in water at 25 °C

Drug	β-CyD	G_1-β-CyD	G_2-β-CyD	$(G_2)_2$-β-CyD
Carmofur [a]	670	600	480	440
Diazepam	210	210	220	210
Nifedipine	190	122	120	90
Nimodipine	480	370	260	230
Nisoldipine	1300	730	630	570
Phenytoin	1100	910	720	700
Prednisolone	3600	2600	2800	2600
Progesterone	13000	17000	15000	17000
Testosterone	7500	8200	8000	8000

[a] In phosphate buffer (pH 3.0).

sidic bond, and their reciprocal plots, respectively. G_1-β-CyD was not hydrolyzed by this enzyme, which is in agreement with the report that pullulanase does not attack the oligosaccharides which have α-1,6 glycosidic bond linking only one glucose unit (Kainuma et al., 1978). Parent β-CyD was not hydrolyzed by this enzyme because it has no α-1,6 glycosidic bond. The K_m values for both substrates were similar with each other (Table 3), while the V_{max} of $(G_2)_2$-β-CyD was about 1.5 times larger than that of G_2-β-CyD, which is in contrast to the case of glucoamylase. The high reactivity of $(G_2)_2$-β-CyD against pullulanase may be due to its multiple reactive points, compared with G_2-β-CyD having only one reactive point, although further study should be done to elucidate their hydrolytic mechanisms.

Inclusion ability of branched β-CyDs

Inclusion abilities of branched β-CyDs against some poorly water-soluble drugs (Table 4) were studied by measuring their solubilizing powers. Fig. 9 shows phase solubility diagrams of nifedipine, nimodipine and nisoldipine, calcium antagonists, with four β-CyDs in water at 25 °C. The low aqueous solubility of the drugs increased linearly with increasing CyD concentrations, showing A_L-type solubility diagrams according to Higuchi and Connors (1965). Other drugs listed in Table 4 gave also A_L-type diagrams with branched β-CyDs, while parent β-CyD showed B_S-type curves with some drugs precipitating solid complexes at higher CyD concentrations (Uekama et al., 1982). The apparent 1:1 stability constant (K_c) of the complexes was calculated from the

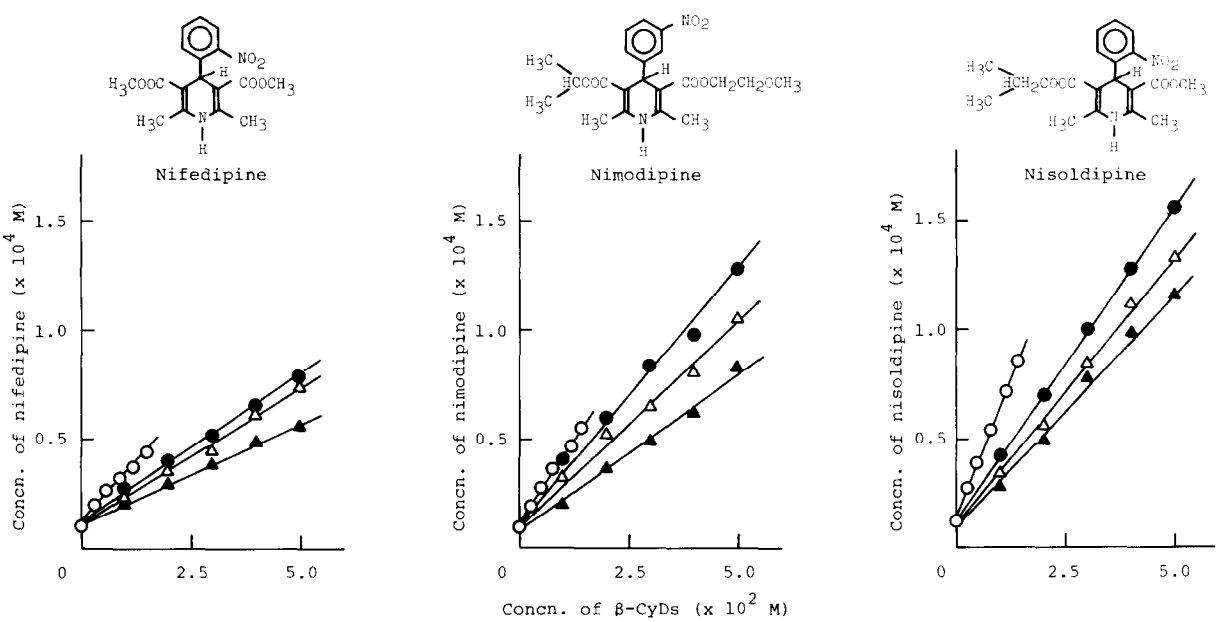


Fig. 9. Phase solubility diagrams of nifedipine, nimodipine and nisoldipine-branched β-CyD systems in water at 25 °C. ○, β-CyD; ●, G_1-β-CyD; △, G_2-β-CyD; ▲, $(G_2)_2$-β-cyD.

initial straight line portion of the solubility diagrams according to the following equation (Higuchi and Connors, 1965) and listed in Table 4.

$$K_c = \frac{\text{slope}}{\text{intercept } (1 - \text{slope})}$$

The K_c value decreased generally in the order of: β-CyD > G_1-β-CyD > G_2-β-CyD > $(G_2)_2$-β-CyD, which may be due to steric hindrance of glucose or maltose residues attached to the primary hydroxyl group of β-CyD. However, the affinity of steroidal drugs and diazepam to branched β-CyDs was comparable to those to parent β-CyD. It is apparent that branched β-CyDs are good solubilizers for poorly water-soluble drugs, compared to parent β-CyD, since their intrinsic aqueous solubility is much higher than that of parent β-CyD and no solid complexes precipitate even at higher CyD concentration.

Among the branched β-CyDs, G_1- and G_2-β-CyDs may be particularly useful to parenteral preparations because of the high resistance of the enzymatic degradation and high solubilizing ability.

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