Inhibitory effects of cyclodextrins by inclusion on the catalytic activity of glycyrrhizinate for the hydrolysis of a nonionic ester surfactant

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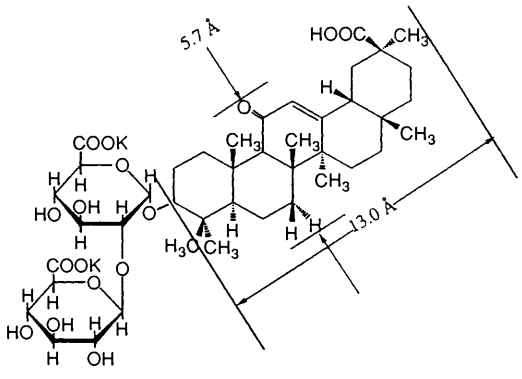
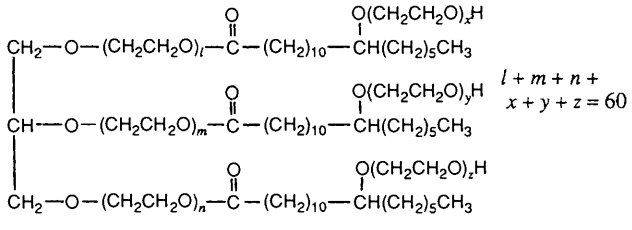
α-, β- and γ-Cyclodextrins (CyDs) and related derivatives have been investigated for their inhibitory effects on the catalytic activity of dipotassium glycyrrhizinate (GK2) in the hydrolysis of polyoxyethylene(60) hydrogenated castor oil, a non-ionic ester surfactant (HCO-60). The activity of GK2 was found to be completely inhibited in the presence of an equimolar amount of γ-CyD owing to the formation of a 1:1 host–guest complex. β-CyD behaved similarly. However, neither inhibition nor complexation was observed in the case of α-CyD. Circular dichroic spectroscopy, using the absorption at 340 nm due to the enone chromophore of GK2, was found to be a useful means to study the details of inclusion of GK2 by CyDs.

Dipotassium glycyrrhizinate (GK2) serves as an anionic surfactant as well as an anti-allergic and anti-inflammatory drug used, for instance, in eye lotions and injectable formulations. Polyoxyethylene(60) hydrogenated castor oil (HCO-60) is added to GK2 as an effective, safe solubilizing



Interatomic distances in GK2



HCO-60

agent for oil-soluble ingredients. However, we have recently found that GK2 catalyses the cleavage of the ester bond of HCO-60 to yield harmful 12-hydroxystearic acid,[1,2] which may be responsible for anaphylatic shock.[3] Our mechanistic study has disclosed that the catalysis by GK2 is due to the presence of the carboxy groups which protonate the ester carbonyl group *via* hydrogen bonding to activate the ester. Such catalysis by hydrogen bonding would be inhibited if the contact of GK2 with the ester bond is prohibited. For this purpose, cyclodextrins (CyDs) appear to be an ideal additive, since they are harmless and their versatility in forming complexes with various substances has been widely recognized to have potentially useful applications in the chemical, food and pharmaceutical industries.[4,5] CyDs have a cavity capable of accommodating a wide variety of organic and inorganic compounds in aqueous solution through hydrophobic and/or van der Waals interactions. In such complexes the hydrophobic portion of the guest molecule makes contact with the apolar wall of the CyD; the triterpenoidal skeleton in GK2 constitutes the hydrophobic portion and would be included in the CyD cavity and thus kept apart from the ester bond.

This paper describes how the catalytic activity of GK2 for the hydrolysis of HCO-60 is completely inhibited by the addition of γ-CyD and that the inhibition is due to the inclusion of the triterpenoidal portion of the GK2 molecule, based on reaction kinetics, circular dichroism (CD) spectroscopy and empirical force field calculations.

Experimental

Materials

α-, β- and γ-CyDs were purchased from Tokyo Kasei Co. and used as received. Heptakis(2,6-*O*-dimethyl)-β-cyclodextrin (DM-β-CyD) and heptakis(2,3,6-*O*-trimethyl)-β-cyclodextrin (TM-β-CyD) were obtained from Tokyo Kasei Co. Heptakis(2,6-*O*-dihydroxypropyl)-β-cyclodextrin (HP-β-CyD) and octakis(2,6-*O*-dihydroxypropyl)-γ-cyclodextrin (HP-γ-CyD) were obtained from Aldrich and used without further purification. GK2 was obtained from Maruzen Seiyaku Co., Hiroshima, Japan and used as received. Reagent grade HCO-60 was obtained from Nikko Chemicals Co., Tokyo, Japan and used as received. 9-Anthryldiazomethane (ADAM) was obtained from Funakoshi Co., Tokyo, Japan and used as received. Tetrahydrofuran (THF) was purified according to the standard methods. Commercially available buffer reagents including citric acid were extra pure. Distilled deionized water was used to prepare the solutions.

Kinetic procedure

The kinetics of hydrolysis of HCO-60 were measured by incubating citrate-buffered solutions of HCO-60, GK2 and a CyD at a constant temperature 70 ± 0.5 °C and the desired pH 4.9. Product analyses were performed by employing fluorescent HPLC analysis of 9-anthrylmethyl 12-hydroxystearate which was derived from the stearic acid produced and the ADAM reagent according to our previously reported method.[1]

CD spectral measurements

CD spectra were recorded on a JASCO J-720 spectropolarimeter.

Computer aided molecular design (CAMD) calculations

Computational molecular mechanics calculations of the stabilization energy of the host–guest complex of GK2 with γ-CyD was performed with the aid of a CAMD system carrying the software package of CHARMm Ver22/QUANTA Ver3.3 (Molecular Simulating Inc., US) on the hardware of INDIGO R-4000 (Silicon Graphics Corporation, US).

Results and discussion

Effects of changing CyD concentration on the hydrolysis of HCO-60

The hydrolysis was performed at pH 4.9 in 0.1 mmol dm^{-3} citrate buffer (mmol = 10^{-3} mol) and 70 °C for 10 days with [GK2] = 1.11 mmol dm^{-3} and [CyD] = 0.0–20.0 mmol dm^{-3}. The addition of CyDs to the reaction solutions made no difference to the kinetic and product features except the rate. The results thus obtained are shown in Fig. 1. As can be seen in Fig. 1, changing the α-CyD concentration had only a slight effect on the hydrolysis conversion, *i.e.* α-CyD has essentially no effect on the catalytic activity of GK2. On the other hand, the hydrolysis was greatly suppressed by the addition of β-CyD and γ-CyD. In particular, addition of an equimolar amount of γ-CyD completely inhibited GK2 activity. These results strongly suggest that the reduction is due to the inhibition by CyDs through host–guest complexation, *i.e.* the cavity size of α-CyD is too small while that of γ-CyD is ideal for the inclusion of GK2. This view was further supported by the following circular dichroic study.

Effect of changing CyD concentration on the CD spectra of GK2

According to our previous report,[1] the CD intensity at 340 nm of GK2 due to the enone chromophore is very sensitive to variation of the solvent polarity and would afford detailed information about the GK2 included in the hydrophobic CyD cavities.

The results shown in Fig. 2 show that the effect of varying α-CyD concentration on the CD intensity at 340 nm of GK2 is negligible. For all the other CyDs, decreasing intensities with increasing CyDs were observed up to saturation. There is a remarkable resemblance between the saturation curves of Figs. 1 and 2. The saturation curves in Fig. 2 could be analysed on the basis of eqn. (1), assuming the formation of a 1:1 host–guest

$$(I_0 - I) = KI_\infty(\mathrm{CyD})/(1 + [\mathrm{CyD}]) \qquad (1)$$

complex between GK2 and CyDs, where K and I are the association constant and the CD intensity, respectively.

It is interesting to compare the curves of the β-CyD series; from Benesi–Hildebrand plots of the curves for TM-β-CyD, β-CyD and DM-β-CyD, K values of 9.2, 470 and 2300 dm^3 mol^{-1}, respectively were obtained. At least two factors seem to be responsible for the magnitude of the K values. The first factor is the effect of free hydroxy groups, the presence of which appears to be required for the stabilization of the complex, since the K value of the permethylated TM-β-CyD is much smaller than

Table 1 Computer-aided molecular design analysis: stabilization potential energies of GK2–CyD complexes

CyD	Stabilization energy/[a] kcal mol^{-1}
TM-β-CyD	93
DM-β-CyD	99
α-CyD	105
HP-β-CyD	112
β-CyD	114
γ-CyD	123
HP-γ-CyD	132

[a] Stabilization energy = the energy of the complex − the sum of energies of the two components GK2 and the CyD in question.

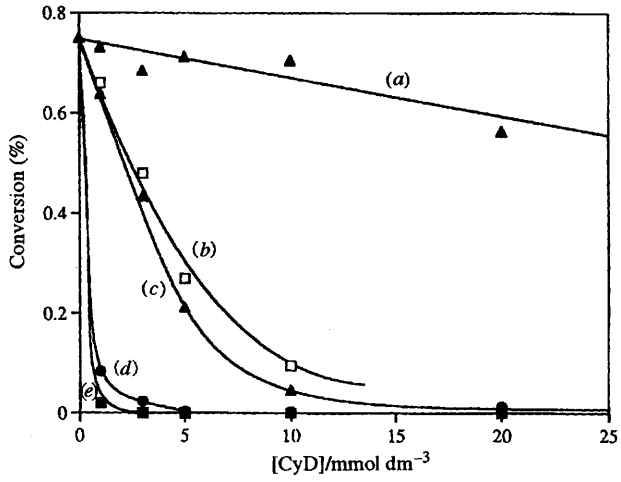


Fig. 1 Inhibition effects of CyDs on the extent of the hydrolysis of HCO-60 by GK2; (*a*) α-CyD, (*b*) β-CyD, (*c*) DM-β-CyD, (*d*) γ-CyD, (*e*) HP-γ-CyD; pH 4.9; 70 °C; 10 days; [HCO-60] = 1.43 mmol dm^{-3}; [GK2] = 1.11 mmol dm^{-3}

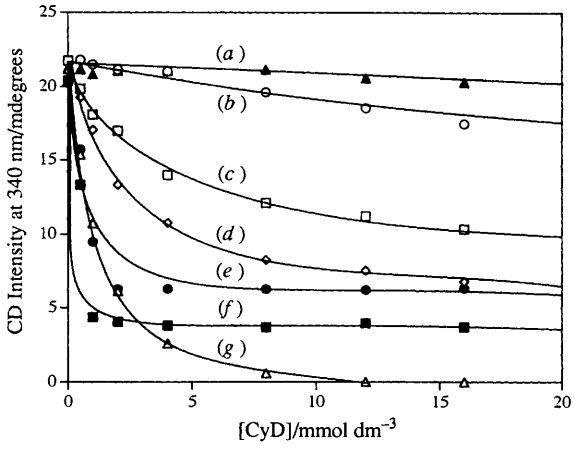


Fig. 2 Saturation plots of the CD intensity *vs.* CyD concentration; (*a*) α-CyD, (*b*) TM-β-CyD, (*c*) β-CyD, (*d*) HP-β-CyD, (*e*) γ-CyD, (*f*) HP-γ-CyD, (*g*) DM-β-CyD, pH 4.9, 20 °C; [GK2] = 1.0 mmol dm^{-3}

those of the other two having free hydroxy groups, β-CyD and DM-β-CyD. There has been recent evidence to support the fact that intermolecular hydrogen bonding interactions are possible even in water when a hydrophobic environment is constructed in the vicinity of the hydrogen bonds;[6] for example, Kano *et al.* have reported hydrogen bonding between the CO^{2-} groups of bilirubin and the secondary OH groups of β-CyD.[7]

However, the observed stability order is not in accord with the calculated order given in Table 1, which shows a decrease with increasing methylation or with a decreasing number of hydrogen bonding interactions: β-CyD > DM-β-CyD > TM-β-CyD. Obviously, the hydrogen bonding is not the compelling

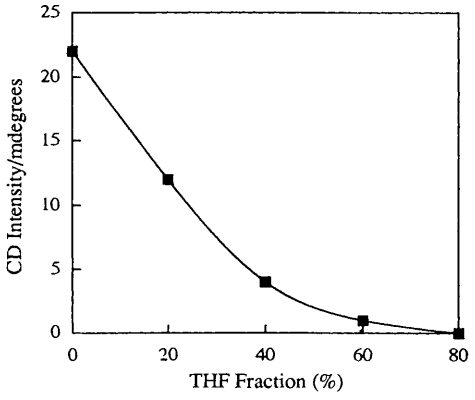


Fig. 3 Variation of the CD intensity *vs.* % fraction of THF; [GK2] = 1.0 mmol dm^{-3}

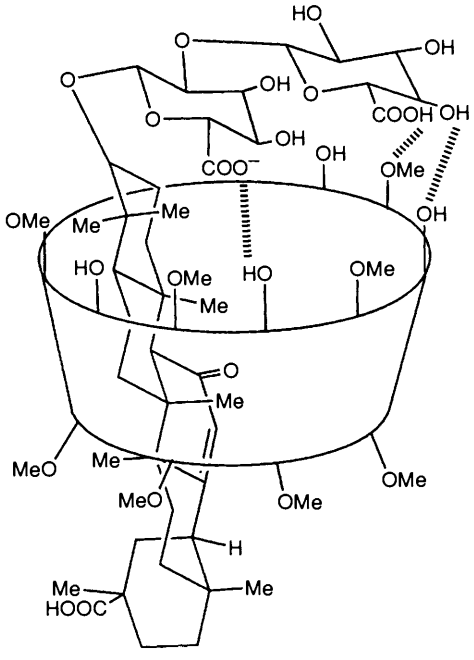


Fig. 4 A conceptual structure of the GK2–DM-β-CyD inclusion complex. For simplicity arbitrarily selected hydrogen bondings only are shown.

factor in determining the observed stability in water. Generally, in calculations where the role played by the solvent is neglected, the contribution of intermolecular hydrogen bonding interactions will be greatly exaggerated. Therefore, another factor is required to account for the reversal in stability between β-CyD and DM-β-CyD. We would expect to find an appreciable contribution of van der Waals interactions and, to a lesser extent, hydrophobic interactions to the highest stability of DM-β-CyD among the three, because the methyl substitutions at the O(2) and O(6) positions in β-CyD make the hydrophobic cavity 40% deeper and thereby enhance the inclusion ability.[8] Moreover, the DM-β-CyD still retains unmethylated hydroxy groups which allows for a number of hydrogen bonds at the secondary hydroxy rim: the secondary importance of the hydrogen bonds can be supported by the finding that, with a guest system having no substituents, such as OH and COO^- moieties, to form hydrogen bonding, there is little or no significant difference in inclusion ability between partially methylated DM-β-CyD and fully methylated TM-β-CyD.[9]

Meanwhile, we have found that the CD intensity arising from the enone chromophore of GK2 is a criterion for the polarity of the microscopic environment around the enone groups; as revealed by Fig. 3, which was previously reported,[1] in a mixed solvent of THF–water the CD intensity decreased monotonically as the % content of THF increased, indicating that the

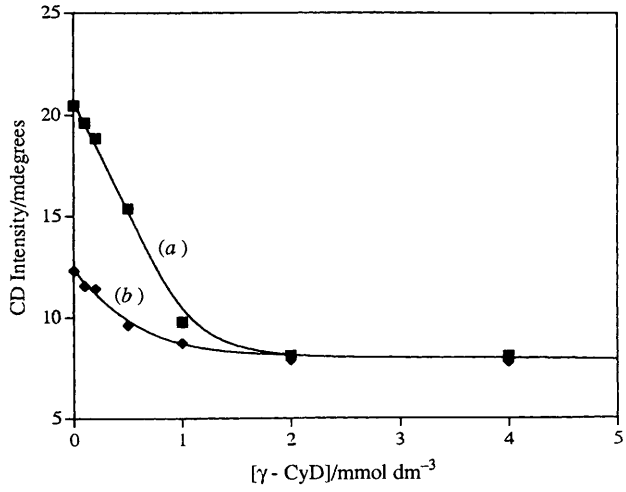


Fig. 5 Plots of the CD intensity *vs.* γ-CyD concentration in the (*a*) absence and (*b*) presence of 1.43 mmol dm^{-3} HCO-60; pH 4.9; [GK2] = 1.0 mmol dm^{-3}

intensity is higher in a more polar solvent and lower in a less polar solvent. Of course, one must be aware that the polarity is a complex function of the orientation and depth of inclusion of the enone chromophore. The zero I_∞ value for DM-β-CyD implies that the enone chromophore fits in the highly apolar part of the interior of the cavity, where the polarity corresponds to at least 80% THF–water solvent. A conceptual model for such a complex is depicted in Fig. 4 in which it is assumed that the end of the triterpenoid portion penetrates the cavity toward the narrower face, while the sugar portion forms hydrogen bonds with the O(3)H groups on the wider face of the CyD.

The saturation curve of γ-CyD in Fig. 2 is an indication of the formation of an unusually strong complex. Since γ-CyD shows a particularly strong inhibitory power in the hydrolysis of HCO-60, a more detailed examination of the CD spectra was carried out both in the absence and presence of HCO-60. The results are shown in Fig. 5. The plots of *I vs.* γ-CyD concentration in the absence of HCO-60 again indicate the formation of a very strong complex in the fashion of a 1:1 stoichiometric ratio of the host–guest, *i.e.* I_∞ was attained at an equimolar amount of host and guest. The corresponding curve in the presence of HCO-60 reveals that the starting intensity is much lower than in its absence, but the intensity of the saturation level is the same in both cases. The lower intensity in the presence of HCO-60 is due to the incorporation of GK2 into the hydrophobic domain of a micelle of the HCO-60; incidentally, HCO-60 which has a critical micellar concentration as low as 2.63×10^{-2} mmol dm^{-3}, forms spherical or nearly spherical micelles at a concentration of 1.43 mmol dm^{-3}.[10] Nevertheless, it is important to notice that the saturation of intensity occurs at the same stoichiometry and with the same intensity in both the absence and presence of HCO-60. This fact implies that the complexation of GK2 and γ-CyD is much stronger than that with HCO-60. However, the non-zero I_∞ value is determined solely by the polarity around the enone group.

Mechanism of inhibition of GK2 activity by CyDs: empirical force field calculations for complexation of GK2 with CyDs

The above results strongly suggest that the inhibition of the catalytic activity of GK2 by CyDs is due to the separation of the catalyst GK2 and the substrate HCO-60 by inclusion of the former. This view was further supported by empirical force field calculations for the complex of GK2 with various CyDs. Values of the calculated stabilization energies are listed in Table 1. The most stable structures are essentially the same for all complexes as proposed in Fig. 4; the triterpenoidal head directs toward the

primary hydroxy face and the sugar tail directs toward the secondary hydroxy face. GK2 is included in the same orientation but with slightly different disposition depending on the CyDs. The TM-β-CyD complex is the least stable among the CyDs examined. Unlike the results of the CD and hydrolysis studies, DM-β-CyD affords a less stabilized complex than the parent β-CyD. This is due primarily to a decrease in the number of hydrogen bonds by methyl substitution.

Higher stabilization energies are obtained for γ-CyD and HP-γ-CyD than for β-CyD and α-CyD, in accord with the relative stability sequence observed in the CD-spectral studies (Fig. 2). It is obvious that an increasing number of hydrogen bonds formed between the GK2 and the CyDs is not the cause of the higher stabilization energies, since seven hydrogen bonds are possible for β-CyD, but only four for γ-CyD and one α-CyD. The γ-CyD cavity with a 7.5–8.3 Å diameter seems to allow for a more favourable maximum van der Waals contact of the triterpenoidal part with the cavity wall than does the β-CyD cavity with a 6.0–6.4 Å diameter; probably, the flexibility, *i.e.* the conformational freedom of the big γ-CyD ring, allows the cavity wall to deform to give a best-fit to the surface of the included GK2 molecule and thereby might maximize the attractive interactions.[11]

In conclusion, it has been found that the undesirable catalytic activity of GK2 toward HCO-60 could be completely suppressed by using γ-CyD or its analogues as inhibitors. The inhibition has been shown to be owing to the formation of stable host–guest complexes. Finally, GK2 was found to be a sensitive probe for studying the microenvironment of the binding site of CyDs and may have some potentially useful applications for other systems.

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Paper 5/03433G
Received 3*th May* 1995
Accepted 23*rd January* 1996