

MOLECULAR INTERACTION OF PHLORIZIN AND CYCLODEXTRINS

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The molecular interaction between phlorizin and cyclodextrin(CD) in aqueous solution is studied using the NMR spectroscopic method. From the chemical shifts of the ^{13}C and ^1H signals, it is concluded that a phlorizin molecule enters into the hydrophobic space of β -CD or γ -CD and makes a loose molecular complex with β -CD or γ -CD. The fact that the H-D exchange reaction in phlorizin is suppressed by β -CD or γ -CD supports the above conclusion. On the other hand, it is found that a phlorizin molecule does not enter into the inner space of α -CD and interacts with α -CD locally as in the case of amylose.

1. Introduction

Phlorizin(Phz) is known to be a glucose-transport-inhibitor at the brush border membrane in the kidney[1]. Although separation and isolation of glucose transporter from kidney have been examined[2], details of the mechanism of the glucose transport have never been explained. In the outer side of the brush border membrane, many filaments have been observed and the diameter of the filaments are known to be 20-30 Å[3]. These filaments are called the sugar coat and the chemical component of the sugar coat is considered to be glycoproteins. These filaments seem to act as a detector of the glucose which is reabsorbed from urine to blood. If Phz exists in urine, filaments can preferably interact with Phz instead of glucose. In this way, glucose transport is presumably inhibited by Phz.

We present the results of NMR spectroscopic studies about the molecular interaction of Phz and cyclodextrins(CDs) in aqueous solutions. Although CDs are composed of only plural glucoses which link with $\alpha(1-4)$ bonding, in this report, they are regarded as a simple model of the sugar coat. CDs have a hydrophobic space which resembles a bucket without a bottom. The diameter of the hydrophobic space increases with the increase of the number of glucose groups[4]. Therefore, CDs can be suitably regarded as a partial model of the filaments. Amylose is a chain polymer composed of plural glucoses which has also a $\alpha(1-4)$ linkage. In this research, the molecular interaction of Phz and

amylose is studied in comparison with those of Phz and CDs.

2 Experimental

^{13}C NMR spectra were obtained with a JEOL FX 200 spectrometer and ^1H NMR spectra were obtained with a JEOL GX 400 spectrometer. An aqueous solution of Phz ($1.5\text{--}3.0 \times 10^{-2}$ mol/l) was used as a sample for NMR spectra. External TMS was used as a reference for ^{13}C signals, while in the measurement of ^1H NMR signals, sodium trimethylsilylpropionate- d_4 was used as an internal reference. Phz (Sigma Chemical Company), CDs (Nakarai Chemicals LTD) and amylose (Hayashibara Biochemical Laboratories Inc.) were used without any purification.

3. Results and Discussions

3.1. Solubility

Ten mg of Phz was saturated in 8.7 ml of water at 23°C . As shown in Fig. 1, it was found that an addition of β -CD caused a considerable increase in the solubility of Phz. While the addition of α -CD does not change the solubility of Phz. A small increase of solubility of Phz was observed when γ -CD was added to Phz. Amylose has a very low solubility in water, and no effect on the solubility of Phz was observed by the addition of amylose.

These solubility changes strongly suggest that a molecular interaction between Phz and β -CD or γ -CD exists, while any similar interaction between Phz and α -CD does not exist. Probably, the insertion of Phz into the hydrophobic space of β -CD and γ -CD increases the solubility of Phz.

3.2. ^{13}C NMR Signals

Fig. 2 shows a ^{13}C NMR spectrum of Phz in the condition of complete decoupling. The numbering of the carbons of Phz and the assignment of the signals are also shown in Fig. 2. The two aromatic rings of

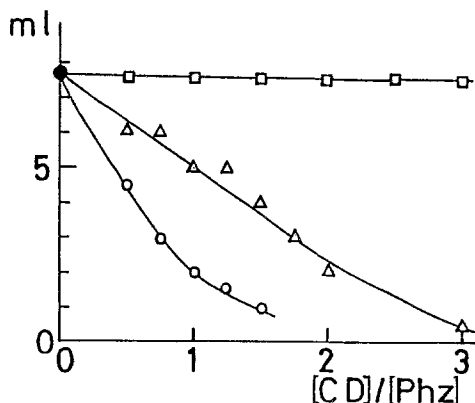


Fig. 1 The volume of water necessary to dissolve 10 mg of Phz when Phz is mixed with α -CD (□), β -CD (○), or γ -CD (△) in several molecular ratios

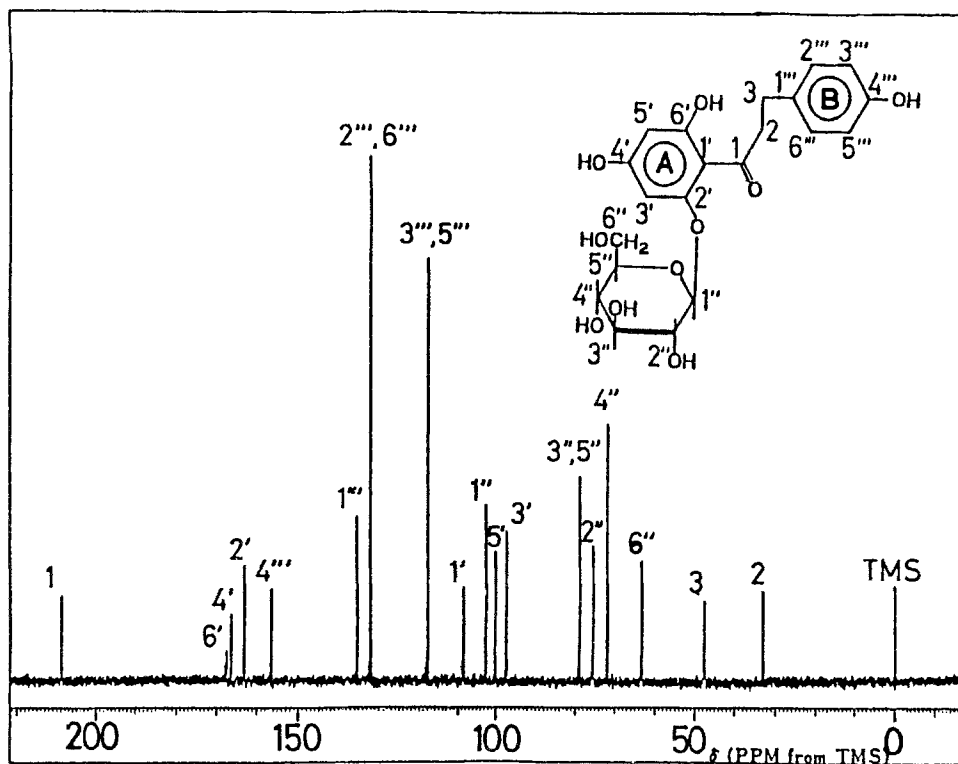


Fig. 2 ¹³C NMR spectrum of Phz.

Phz are named as the A and B rings as shown in Fig. 2. Although CDs are composed of plural glucoses, ¹³C NMR spectra show only six signals. This indicates very fast exchanges among many conformational isomers of the glucoses in CD. Mixing of Phz and CDs causes many changes in the chemical shifts of the ¹³C signals of both Phz and CDs. Fig. 3 shows a comparison of the chemical shifts of the mixture ([Phz]/[CD]=1) with free Phz and free CDs. In the case of the α-CD mixture, only a 0.7 ppm high field shift of C-1 of Phz and a 0.2 ppm high field shift of C-2 of Phz are observed, but other signals of Phz and α-CD remain unchanged. Similar changes are also observed in the case of the amylose mixture. These changes are explained by the existence of intermolecular hydrogen bonding between the carbonyl group of Phz and hydroxyl groups of α-CD or amylose. The diameter of the hydrophobic space of α-CD is too short for the Phz to enter into the space.

However, in the case of the mixture of Phz and β-CD or γ-CD, considerable chemical shift changes are observed in many carbons of both Phz and CDs. In these two cases, the direction of the change differs in many carbons. Thus the molecular configuration of Phz interacting with CD seems to be different in the two cases. Fig. 4 and 5 show the changes of the chemical shifts in several mixing ratios of β-CD and γ-CD to Phz, respectively. Continuous changes of the chemical shifts are

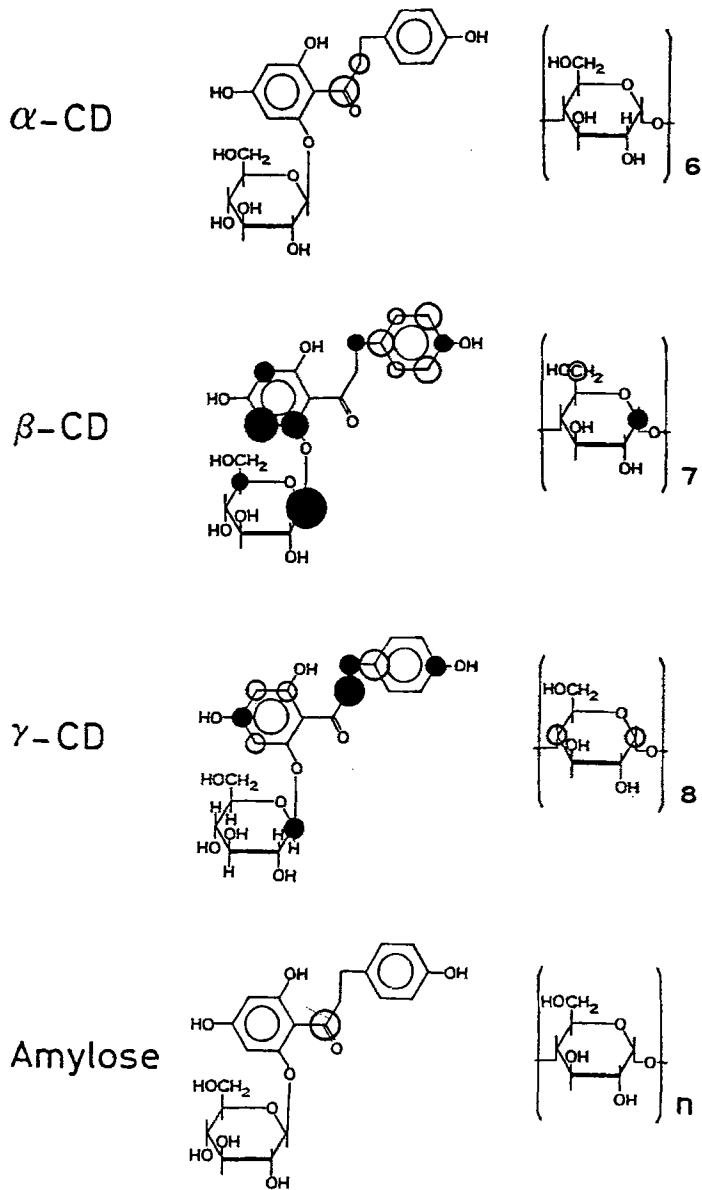


Fig. 3 Comparison of ^{13}C chemical shifts of the mixture ($[\text{Phz}]/[\text{CD}]=1$) with free Phz and free CD. White circles (○) show high field shifts of the mixture and black circles (●) show low field shifts of the mixture. The diameter of the circle shows the relative magnitude of the shifts.

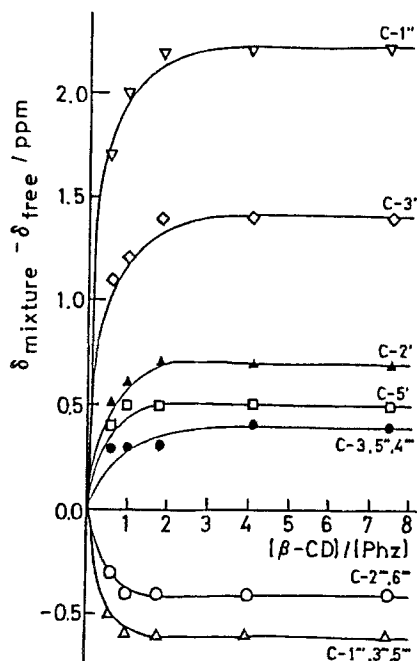


Fig. 4 Changes in ^{13}C chemical shifts of β -CD mixtures.

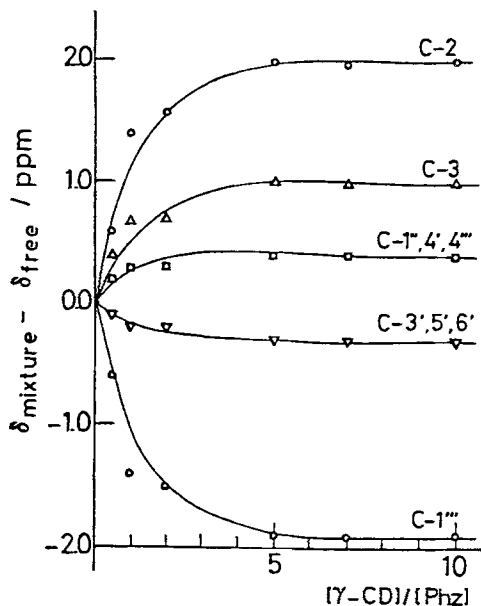


Fig. 5 Changes in ^{13}C chemical shifts of γ -CD mixtures.

observed even at the ratio larger than 1 in both cases. This suggests that the insertion reaction of Phz into the inner space of CD is reversible and that the equilibrium favours the complex form at the large mixing ratio.

3.3. ^1H NMR Signals

In the ^1H NMR spectra of Phz and CDs, only protons which bond directly to carbon atoms are definitely assigned. By an addition of CD to Phz, some ^1H signals shift to higher field. The comparison of chemical shifts of the mixture ($[\text{Phz}]/[\text{CD}]=1$) with those of free Phz and free CDs is shown in Fig. 6. Protons in the glucose ring of Phz, except $\text{H}-1''$, are excluded from the analysis, because the signals are complicated in the mixture by the strong signals of CDs. No changes are observed in the cases of the α -CD mixture and the amylose mixture. However, in the cases of the β -CD and γ -CD mixtures, high field shifts are observed with both Phz and CD. Fig. 7 and 8 show the changes in the ^1H chemical shifts of Phz by additions of β -CD and γ -CD, respectively, in several mixing ratios. The changes in the β -CD mixture are relatively large compared with those in the γ -CD mixture. Thus in the case of the β -CD mixture, hydrogen atoms of the Phz molecule seem to be

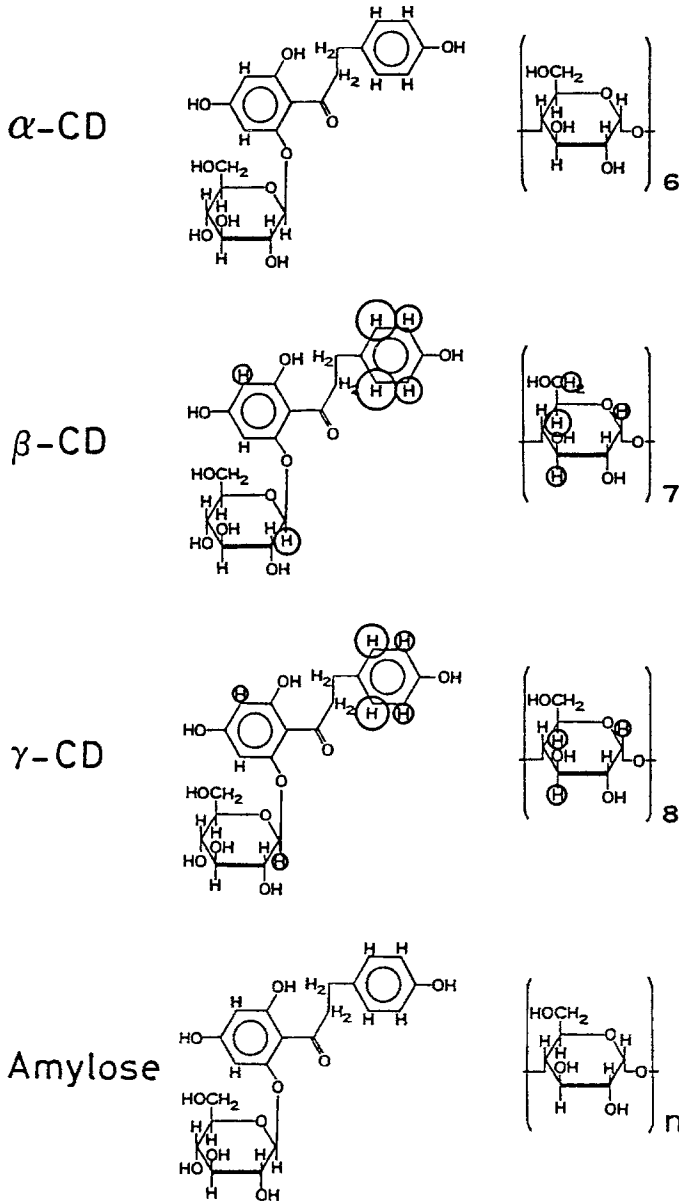


Fig. 6 Comparison of 1-H chemical shifts of the mixture ([Phz]/[CD]=1) with free Phz and free CD. Circles show high field shifts of the mixture and the diameter of the circles show the relative magnitudes of the shifts.

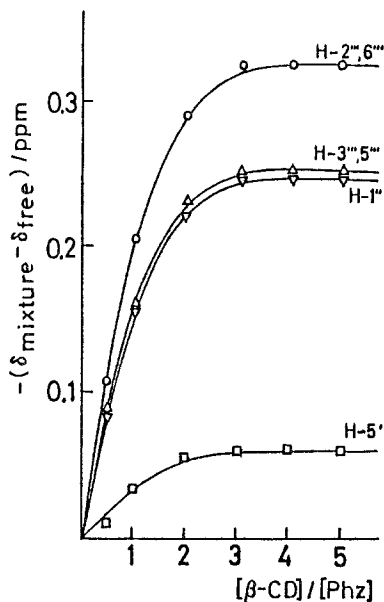


Fig. 7 Changes in 1-H chemical shifts of β -CD mixtures.

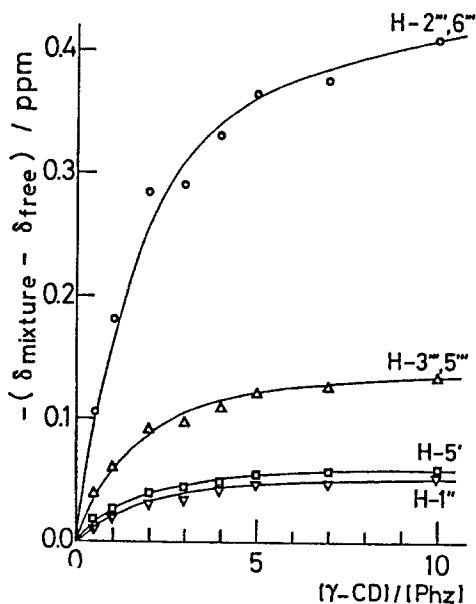


Fig. 8 Changes in 1-H chemical shifts of γ -CD mixtures.

nearer to atoms of the CD molecule than in the case of the γ -CD mixture. It is also evident that protons which show the large changes of chemical shift belong to the B aromatic ring. This suggests the B aromatic ring enters into the inner space of β -CD or γ -CD. Continuous changes of the chemical shifts are again observed at the ratio larger than 1 as observed with the ^{13}C signals. This fact supports the discussion in the preceding section.

3.4. H-D Exchange Reaction

The H-3' and -5' signals of Phz are observed as doublets at 6.26 and 6.12 ppm, respectively, a few hours after dissolving Phz in D_2O . 7 Days after the dissolution, the H-3' signal becomes a singlet and the signal intensity of H-5' decreases drastically, as shown in Fig. 9. These facts are explained by H-D exchange of H-5'. A Phz molecule in D_2O can contact freely with the solvent and the exchange reaction progresses quickly. However, the H-D exchange is repressed in both cases of β - and γ -CD mixtures. Fig. 10 shows the time dependency of the H-D exchange in H-5' when the solution is left at 20°C . Mixing of Phz with α -CD or amylose results in no repression of the H-D exchange. These facts distinctly indicate that the A aromatic ring enters into the inner space of β - or γ -CD, which causes the slow H-D exchange reactions,

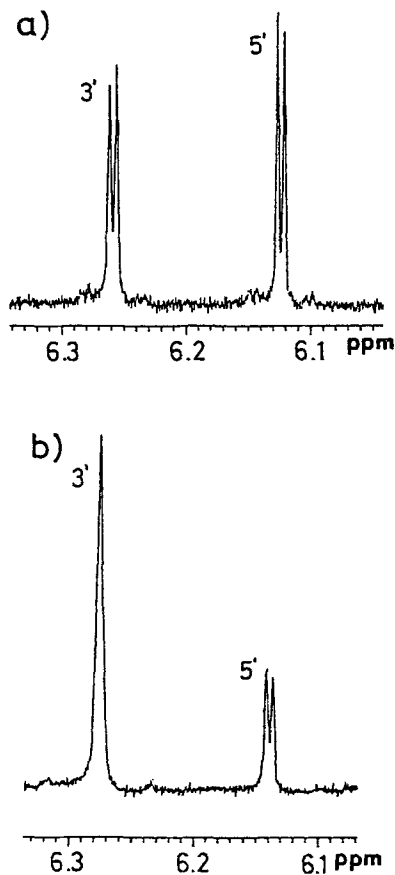


Fig. 9 H-3' and H-5' signals of Phz, a) a few hours after dissolving, b) 7 days after dissolving.

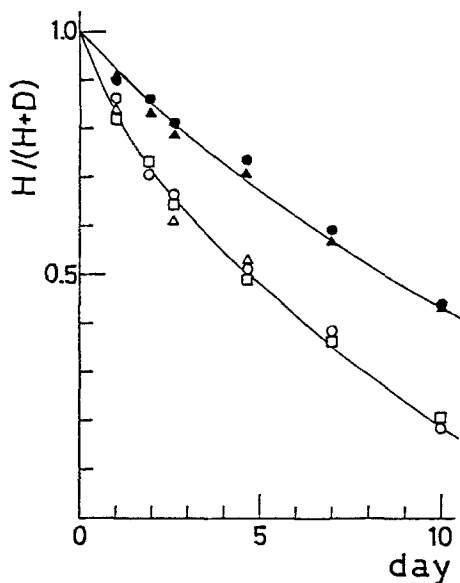


Fig. 10 The time dependence of the H-D exchange reaction of H-5' at 20°C. Solutions of Phz only (○), Phz+α-CD(Δ), Phz+β-CD(●), Phz+γ-CD(▲), and Phz+amylose (□) are compared. Mixing ratio equals 1 in all cases

because the insertion into the inner space of CD disturbs the H-5' contact with D₂O. On the other hand, in the case of the α-CD mixture, free Phz out of the inner space of α-CD can contact freely with the solvent. In the case of the amylose mixture, Phz reacts also with the solvent freely, because amylose has no hydrophobic space. These facts directly indicate that at least the A aromatic ring of Phz enters into the inner space of CD in the cases of β-CD and γ-CD.

4. Conclusions

From the results of ¹³C and ¹H NMR spectral analyses, the

following very important facts were found. At least, the A aromatic ring of Phz enters into the hydrophobic space of β -CD and γ -CD. Since the large changes of the 1-H and 13-C chemical shifts are observed with many protons and carbons in the B aromatic ring of Phz, the possibility of the insertion of the B aromatic ring of Phz into the inner space of β -CD and γ -CD is considerably large. Particularly, the large changes of the 1-H chemical shifts are observed only with protons of the B aromatic ring in both cases of the β -CD mixture and the γ -CD mixture. The Phz molecule does not enter into the inner space of α -CD, probably because the diameter of the inner space of α -CD is too small to accommodate Phz.

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