Aqueous Solubility Enhancement of Some Flavones by Complexation with Cyclodextrins

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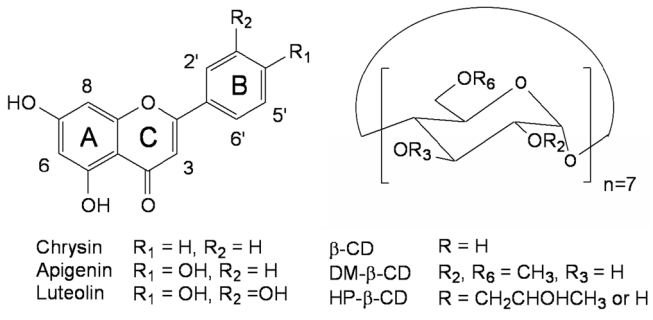
The inclusion complexes of cyclodextrins (CDs) with flavones in aqueous solution were investigated by phase solubility measurements. The effect of β-cyclodextrin (β-CD), heptakis (2,6-di-*O*-methyl) β-cyclodextrin (DM-β-CD) and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) on the aqueous solubility of three flavones, namely, chrysin, apigenin and luteolin was investigated, respectively. Solubility enhancements of all flavones obtained with three CDs followed the rank order: HP-β-CD > DM-β-CD > β-CD, and besides, CDs show higher stability constant on luteolin than that on others flavones. ^{1}H-nuclear magnetic resonance (NMR) spectroscopy and molecular modeling was used to help establish the model of interaction of the CDs with luteolin. NMR spectroscopic analysis suggested that A-C ring, and part of the B ring of luteolin display favorable interaction with the CDs, which was also confirmed by docking studies based on the molecular simulation. The observed augmentation of solubility of luteolin by three CDs was explained by the difference of electrostatic interaction of each complex, especially hydrogen bonding.

Key Words : Cyclodextrin, Flavone, Luteolin, Host-guest interaction, Solubility

Introduction

Flavonoids, classified mainly into four subgroups: flavone, flavonol, flavanone, isoflavone, are polyphenolic compounds that usually exist in plants as secondary metabolites.[1] They possess strong antioxidative activity as well as other potential effects including anti-inflammatory, anti-cancer, and anti-viral.[2] Owing to their phenolic nature, flavonoids are quite polar but poorly water soluble, and their scarce absorption is well known.[1] These aspects have limited their use in the pharmaceutical field. Thus, we have decided to investigate the supramolecular complexes of some flavones in the cavity of the cyclodextrins to improve their solubility.

Cyclodextrins (CDs) are cyclic oligosaccharides composed of glucopyranose units and adopt a truncated cone structure with hydrophobic cavity. The non-polarity of the interior cavity of the cyclodextrin makes it ideal for solubilizing nonpolar solutes, whereas the polarity of its exterior helps it and its guest to become soluble in water.[3] This property accounts for the great interest in cyclodextrins and it was shown that complex formation can be improved by chemical modifications of native cyclodextrins.[4]



Scheme 1. Schematic representation for the structure of flavones (A) and cyclodextrins (B).

Three flavone aglycones namely chrysin, apigenin and luteolin (Scheme 1) were selected for the inclusion study, in order to analyze the effects of the different hydroxylation degree of ring B on the ability to complex with cyclodextrins. β-Cyclodextrin and two modified cyclodextrins, heptakis (2,6-di-*O*-methyl) β-cyclodextrin (DM-β-CD) and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), were used to increase the solubility of flavones. Flavone-cyclodextrin interactions in solution were investigated by phase-solubility analysis supported by a NMR and computer-aided molecular modeling approach.

Experimental Section

The chrysin, apigenin, luteolin, β-cyclodextrin, heptakis (2,6-di-*O*-methyl) β-cyclodextrin (DM-β-CD), and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) [M.S. = 1] were purchased form Sigma-Aldrich Inc. St. Louis, MO. They were employed without further purification. All other materials were analytical grade, and all water used double-distilled and deionized. UV-vis absorption spectra were obtained with a spectrophotometer UV-Vis (Ultraspec 3100 pro, Amersham biosciences), connected to a PC for data processing (SWIFT II, Amersham biosciences). ^{1}H-NMR spectra were recorded in deuterated dimethyl sulfoxide (DMSO-d_6) solutions. The spectra were obtained at 300 K using a Bruker AMX spectrometer operating at 500 MHz. Molecular

modeling was carried out using the Insight II molecular modeling package (Accelrys, San Diego) un on Pentium PC.

Phase-solubility studies were performed according to the method of Higuchi and Connors.[5] A fixed initial amount of flavones (200 μM), exceeding their solubility, was added to unbuffered aqueous solutions of cyclodextrins (0.0-8.0 mM) in capped vials, then sonicated for 15 min. Vials were sealed to avoid changes due to evaporation and magnetically stirred for 72 h, shielded from light to prevent degradation of the molecules. At the equilibrium (about 72 h), the aliquot from each vial was filtered through a PVDF 0.2 μm filter (Whatman). Each sample was analyzed by spectrophotometry at 286 nm and 362 nm to evaluate the concentration of the flavones dissolved. The apparent stability constants (K_C) of the flavone-cyclodextrin complexes were calculated from the straight lines portion of the phase-solubility diagrams according to Higuchi-Connors equation: K_C = slope/intercept (1-slope).

The molecular structure of β-CD was obtained from crystal structure. DM-β-CD, HP-β-CD, and luteolin were built using the Builder module of the Insight II program by adding to β-CD 14 methyl in position 2 and 6 (DM-β-CD) and 7 hydroxypropyl groups on the primary hydroxyl groups of β-CD as shown by Mura *et al*. (HP-β-CD).[6] The obtained models were optimized using a protocol of 300 steps of conjugated gradients to avoid steric hindrance. After thorough minimization, docking experiments were carried out using "Dock" modules in Insight II and the CVFF force field for docking and scoring.[7] The luteolin was initially set above the center of the cavity of CD with a distance of ~15 Å. During the course of docking simulations, a luteolin could make a maximum translational movement of 3 Å and a maximum rotation of 180° around the *x*, *y*, and *z* axes. Each cycle began with a random change of up to 5 degrees of freedom among them. If the energy of the resulting configuration was within 1,000 kcal/mol of the last accepted one,[8] it was subjected to 100 iterations of conjugated gradient energy minimization. After the energy minimization, the resulting structure was accepted based on the following criteria: (a) an energy check and (b) a root-mean-squared displacement (RMSD) check, which compared the energy and RMSD of the new configuration against those accepted so far. Configurations above 1 kcal/mol energy and within 0.1 Å RMSD of pre-existing ones were discarded to obtain lower energy and to avoid accepting similar configurations. The docking simulations were performed until energy convergence and up to 100 structures obtained. The most stable structure of the complexes was the one with their lowest interaction energy. Interaction energies of the complexes were calculated as the difference between the total energy of the complex and the sum of the energies of the single components in a free state.

Results and Discussion

The stoichiometric ratios and stability constants describing were obtained by measuring the changes in UV-vis absorbance of the flavones, in the presence of increasing concentrations of the cyclodextrins. Figure 1 shows that the absorption intensities of chrysin and apigenin increased by increasing the HP-β-CD concentration. The insets in the same figure represent show the phase-solubility diagrams obtained for HP-β-CD with chrysin and apigenin, respectively. The solubility of two flavones is enhanced by the presence of the HP-β-CD. Other combinations of cyclodextrins (β-CD and DM-β-CD) and flavones (chrysin and apigenin) also show the same phases. However, the solubilizing efficiency is lower than HP-β-CD complex. The phase-solubility diagram is a widely accepted method for evaluation of the effect of CD complexation on the guest solubility. The 1:1 flavonoid/cyclodextrin complex is the most common type of association where a single flavonoid is included in the cavity of one cyclodextrin, with a stability constant K_C for the equilibrium between the free and associated species.[9] Furthermore, since the slope of the diagram is lower than unit, the stoichiometry of the complexes was assumed to be 1:1.[5] The stability constants K_C were calculated from the straight-line portion of the phase-solubility diagram.

Table 1 shows the stability constants of each complex.

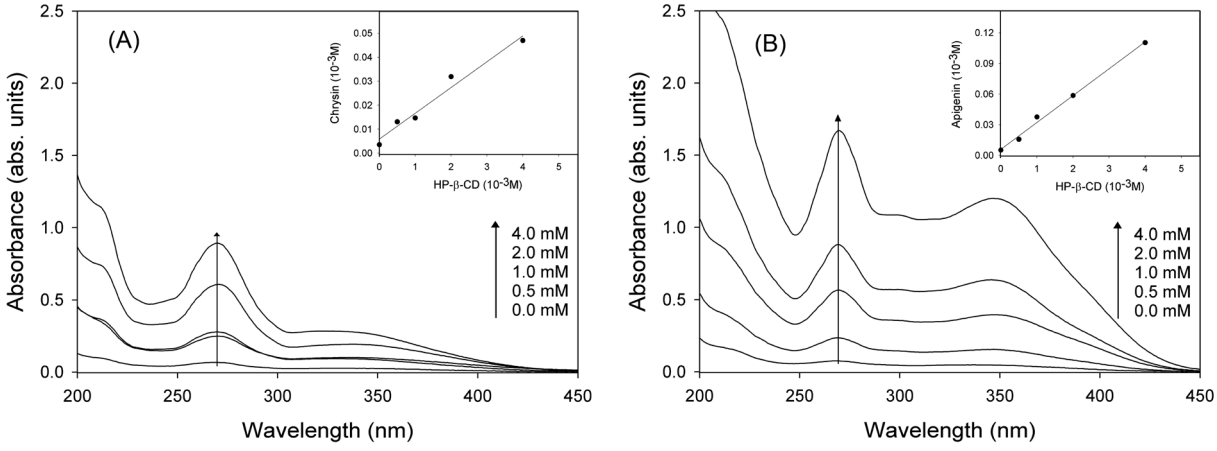


Figure 1. UV-vis absorption spectra of chrysin (A) and apigenin (B) in the presence of increasing concentrations of HP-β-CD. In the corresponding insets, phase solubility diagrams of chrysin and apigenin as a function HP-β-CD concentration in water at room temperature are shown.

Table 1. Stability constants of flavones with cyclodextrins in water

Flavonoid	Stability constant K_C (M^{-1})		
	β-CD	DM-β-CD	HP-β-CD
Chrysin	975.6	1,062.1	1,858.5
Apigenin	827.6	1,038.6	4,511.5
Luteolin	2,328.3	4,461.2	51,385.9

Luteolin complexes show higher stability constants than other flavonoid complex. In the case of HP-β-CD, more hydroxyl containing flavone shows higher stability constant followed the rank order: luteolin, apigenin and chrysin. Furthermore, the solubilizing efficiencies of the HP-β-CD, calculated as the ratio between flavones solubilities in 2 mM HP-β-CD aqueous solution and in pure water, resulted clearly greater (about 70.2 for the luteolin) than that of the others (about 9.1 for the chrysin and about 11.5 for the apigenin). Therefore, we focused on the complex formation of cyclodextrin with luteolin. Figure 2(A)-(C) show the UV absorption spectra of luteolin in the presence of increasing concentrations of each cyclodextrins. In particular, a linear increase in solubility of luteolin with concentration of β-CD is observed in all the explored range whereas there is a well defined plateau at 2×10^{-4} M of luteolin for DM-β-CD and HP-β-CD, indicating that at this point luteolin must be fully complexed (Figure 2(D)). HP-β-CD is more efficient solubilizing agent than other cyclodextrins investigated.

In NMR analysis, formation of β-CD inclusion complexes is normally evidenced by changes in chemical shifts. Such chemical shift changes may provide valuable insight into the molecular conformation of the inclusion complexes. Being more sensitive than carbon NMR spectroscopy, proton NMR spectroscopy has been more widely used to characterize inclusion complexes involving cyclodextrins.[10] In the present study, owing to the extremely poor aqueous solubility of flavones, DMSO was used as a solvent, in order to obtain the optimum solubility of both species. The spectra of cyclodextrins are not presented here because of the relatively impure chemical nature and poor spectral resolution of the modified cyclodextrins. Therefore, the present study has only considered the aromatic proton chemical shifts of luteolin which show high stability constant with cyclodextrins.

Table 2 lists the detailed variation of the aromatic chemical shifts of luteolin. The major induced shift is observed for all the A-C ring protons (H3, H6, and H8) and part of B ring proton (H5') of luteolin on all complexes. However, there are some different aspects between stability constant in purely aqueous solvent system (Table 1) and the degree of chemical shifts in DMSO environments (Table 2). This might be due to the effects of DMSO on the complexes.

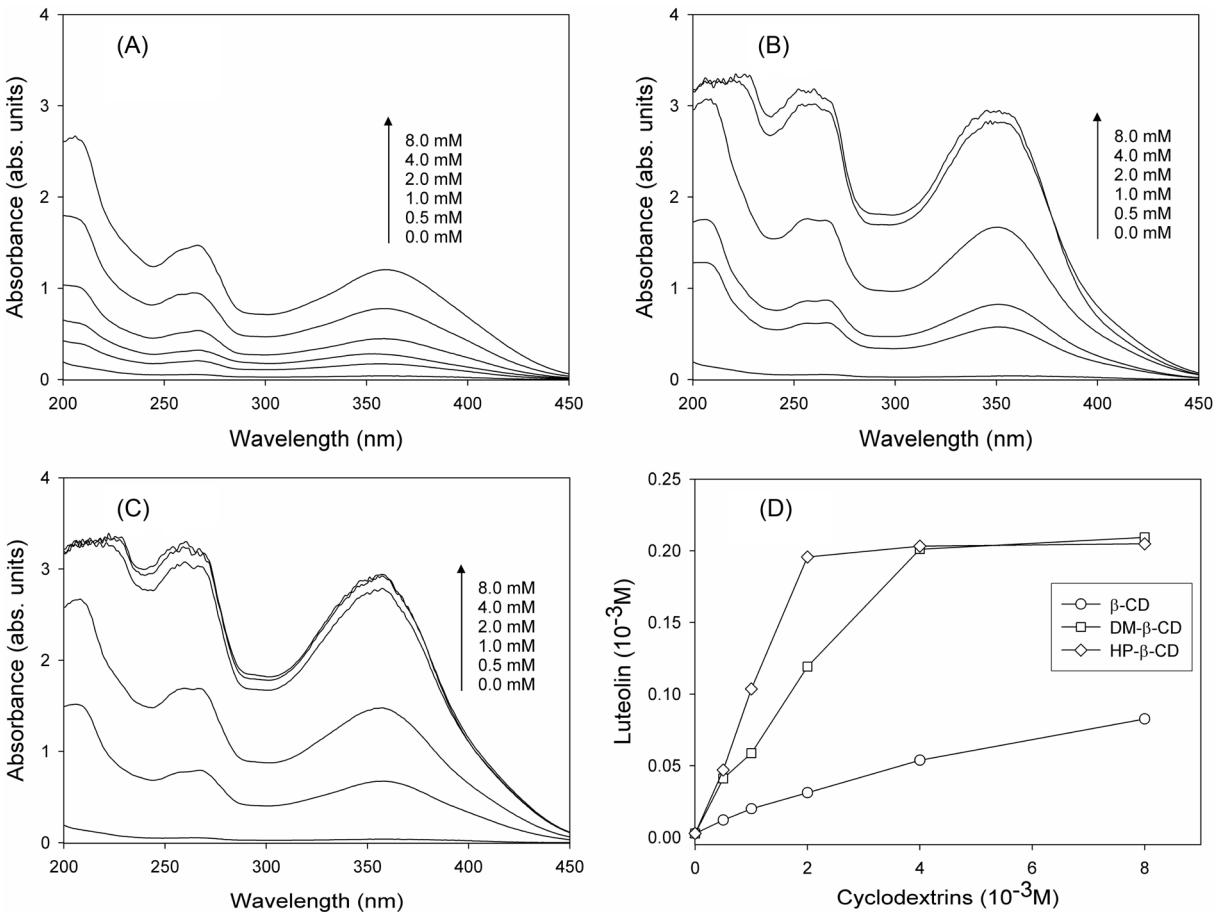


Figure 2. UV-vis absorption spectra of luteolin in the presence of increasing concentrations of: (A) β-CD, (B) DM-β-CD, and (C) HP-β-CD (0.0-8.0 mM). (D) Phase solubility diagrams of luteolin with each cyclodextrins. Key: (○) β-CD, (□) DM-β-CD, (◇) HP-β-CD.

Table 2. ^{1}H NMR shifts ($\times 10^{-2}$) of free luteolin and complexed luteolin

	$\Delta\delta = \delta_{complex} - \delta_{free}$					
	3	8	6	2'	6'	5'
β-CD	1.43	1.33	1.31	0.58	0.80	1.34
DM-β-CD	0.85	0.82	0.79	0.34	0.48	0.80
HP-β-CD	1.46	1.40	0.95	0.60	0.84	1.38

DMSO can form the inclusion complex with β-cyclodextrin[11] and also act as a competitive guest on the formation of an inclusion complex between cyclodextrin and guest.[12] Furthermore, Zheng *et al.* show that 20% v/v DMSO drastically lowered the K_C values of complexes about 20-fold.[13] However, the reducing the binding strength of the complex in water will not significantly alter the basic model of interaction.[13-15] Though DMSO can alter the stability constant of cyclodextrin complexes, we focused on the binding mode of interactions of luteolin.

Based on the results of NMR and phase solubility studies, it can be inferred that the A-C ring and at least part of the B-ring of luteolin exhibit significant interaction with the cyclodextrin cavity. To further investigate the mode of interaction between luteolin and cyclodextrins, a molecular modeling study was performed.[7] The most stable structure from each docking simulation for the 1:1 inclusion complex between cyclodextrins and luteolin are shown in Figure 3. The most stable structure of the complexes was the one with their lowest interaction energy in accepted structures. The main moiety complexed in the cavity of CDs is A-C ring of luteolin in all three cases. The center of mass distances between each cyclodextrin and A-C ring are 1.46, 1.93 and 1.87 Å in β-CD, DM-β-CD, and HP-β-CD, respectively. In the case of B ring, the distance is larger and above 3.48 Å in all three complexes. These indicate that the major interaction moiety of luteolin with CDs is A-C ring, which is agreement with the major chemical shift in Table 2.

Table 3 shows the docking energies between luteolin and cyclodextrins. The total energy of each complex followed in ascending order of HP-β-CD > DM-β-CD > β-CD, which is the same order of stability constants (Table 1). For the van der Waals energies are similar with three cyclodextrins complexes (−43.5, −45.3, and −42.0 kcal/mol in β-CD, DM-β-CD, and HP-β-CD respectively). However, electrostatic energy is quite different and the order is the same with that of stability constants. These result shows that the electrostatic interaction of complexes is a decisive factor for the stabilizing and solubilizing flavones in aqueous environments. Furthermore, the hydrogen bonding of complex is more important than other factors and depicted by dashed line in Figure 3. In the most likely favorable structure of each complex, there are 2, 1, and no hydrogen bonding in HP-β-CD, DM-β-CD, and β-CD complexes, respectively. These data indicate a more stable inclusion complexation of chemically modified β-cyclodextrins than native β-CD and hydroxypropyl moiety of CDs is more effective than dimethyl one to complex with luteolin.

Table 3. Intermolecular energies between luteolin and cyclodextrins

	van der Waals	Electrostatic	Total
β-CD	−43.5	2.2	−41.3
DM-β-CD	−45.3	−7.2	−52.5
HP-β-CD	−42.0	−14.9	−56.9

Our previously study also presented similar finding that molecular modeling could predict accurately the order of stability constants between natural α-, β-, and γ-cyclodextrin complexes with monocylic molecules.[7] This suggests that in the development of new chemically modified cyclodextrins or formulations of cyclodextrin complexes, the cyclodextrin molecules and their complexes can be assessed computationally for the feasibility even before the actual synthesis and experiments are carried out.

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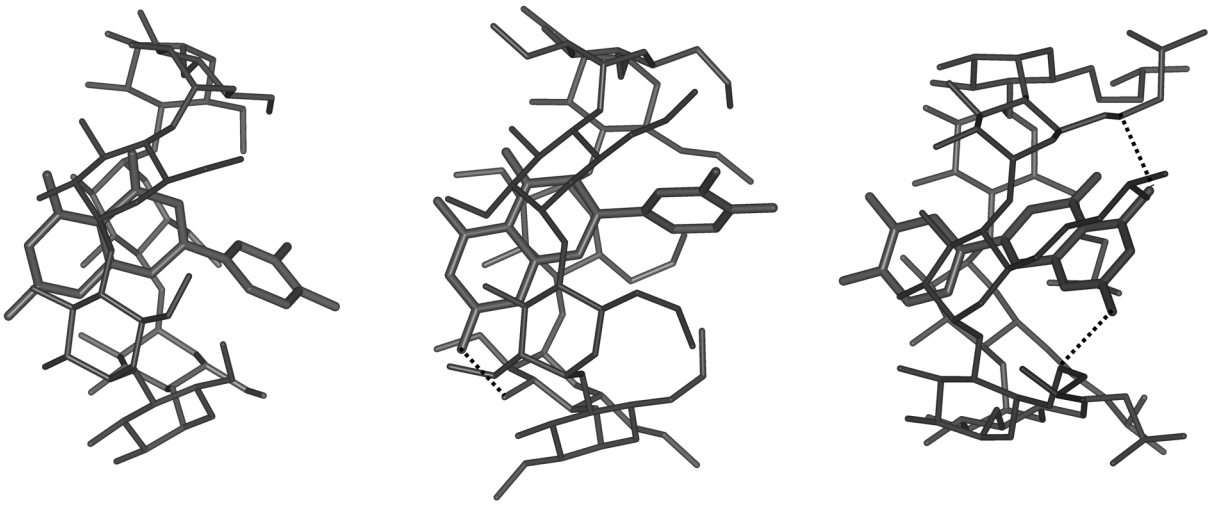

Figure 3. Proposed favorable inclusion complexes of luteolin with β-CD (left), DM-β-CD (middle), and HP-β-CD (right) obtained from molecular docking studies. The dashed lines represent the hydrogen bonding between luteolin and cyclodextrins.

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