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Enhancement of Solubility and Antioxidant Activity of Some Flavonoids Based on the Inclusion Complexation with Sulfobutylether β-Cyclodextrin

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Flavonoids are polyphenolic photochemicals generally found in plants, foods, and beverages. They contribute to plant colors in fruit, leaves providing a wide spectrum of color from red to blue in flowers.[1] Flavonoids have many good physiological activities such as the antioxidant, antitumor, and antibacterial activities which have been a focus of the attention of many researchers.[2] There are four subgroups of flavonoids, flavone, flavonol, flavanone, and isoflavone, according to their chemical structure.[3]

We investigated inclusion complexes of cyclodextrins with three different flavonoids (Figure 1), luteolin, kaempferol, and myricetin, to modulate the solubility and antioxidant activity of flavonoids. Luteolin is a flavone and has been found in many plants, carrots, peppers, rosemary, tea and chocolate.[4] It has physiological functions such as cancer chemopreventive potential, antimicrobial, anti-oxidant activity and anti-inflammatory effects *in vivo* and *in vitro*.[5] Kaempferol and myricetin are classified into flavonol subgroup. Kaempferol isolated from plant and tea has anti-oxidant,[6] antidepressant properties and is known to reduce the heart risk. Myricetin[7] produced by fruits, herbs and vegetable has antioxidant property.[6] These three flavonoids have antioxidant effect[8] or free radical scavenging properties which show the positive effects on the treatment and prevention of a wide range of oxidative-stress associated pathologies, including cancer and heart disease.[9] In spite of these useful physiological properties of flavonoids, they have very poor aqueous solubility where myricetin is soluble in boiling water[7] or alcohol and kaempferol and luteolin are soluble in organic solvent.

Cyclodextrins (CDs) are cyclic oligosaccharides and truncated structure with a hydrophobic hole. They are consisting of six (α-CD), seven (β-CD), eight (γ-CD) or more glucopyranose units linked by α(1-4) glycosidic bonds. CDs have been widely used to improve the solubility of many insoluble or hardly soluble drugs based on the inclusion complexation using their hydrophobic cavities.[10-11] Especially β-CD (Figure 1) is generally most useful which is the most accessible and the lowest-priced. Cyclodextrins may contain one or more entrapped guest molecules though the ratio of host to guest is 1:1 in many cases.[12] There are some reports on the complexation study of some flavonoids such as luteolin,[13] kaempferol[14] or myricetin[14] with β-CD, HP-β-CD or DM-β-CD.

In this report, β-CD and sulfobutylether-β-cyclodextrin (SBE-β-CD,[15] Figure 1) were used to increase the solubility and antioxidant activity of some flavonoids. SBE-β-CD is a negatively charged β-CD derivative prepared by the addition of sulfobutylether group to β-CD. SBE-β-CD has been used one of the most popular β-CD derivatives to improve the ability to solubilize some poorly water soluble molecules.[16] The SBE groups were variably substituted at the 2-, 3-, 6- position of the hydroxyl group of β-CD[17] and the average of total degree of substitution is seven SBE group per β-CD molecule.[15] We think SBE groups attached to CD may positively contribute to the more effective complexation of some flavonoids used in this study comparing with original β-CD.

Figure 2 shows the UV absorption spectra of luteolin in aqueous solution with β-CD and SBE-β-CD, where the absorption intensity of dissolved luteolin is increased by the addition of 4 mM β-CD and SBE-β-CD. The addition of SBE-β-CD

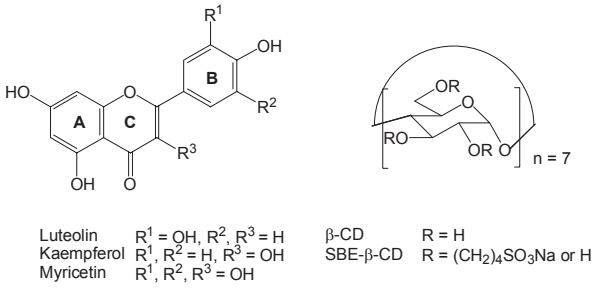

Figure 1. Schematic representation for the structure of flavonoids and cyclodextrins.

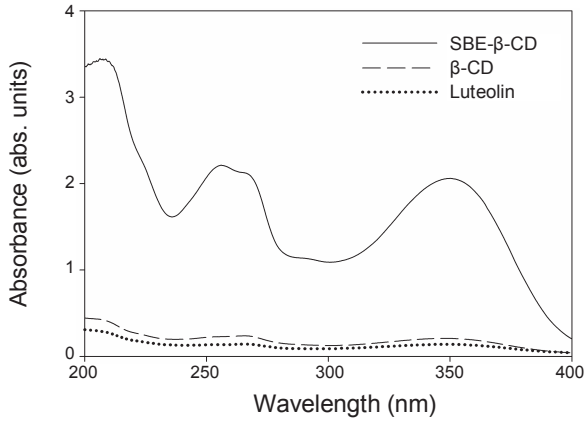

Figure 2. UV-vis absorption spectra of luteolin with and without 4mM β-CD and SBE-β-CD in water at 30 °C.

shows more effective UV absorbance increase than β-CD addition. The absorbance of dissolved luteolin of SBE-β-CD complex is 10.31 fold increase than that of β-CD complex. In the cases of kaempferol and myricetin, similar phenomena were observed (Data not shown). The absorbance of kaempferol and myricetin was increased up to 18.67 fold and to 3.36 fold respectively by adding of 4 mM SBE-β-CD compared with 4 mM β-CD addition. Figure 3 shows the phase solubility diagram of flavonoids with CDs. As the concentrations of added CDs increased, solubility of flavonoids was also increased. Each of luteolin and kaempferol must be fully complexed when 4 mM and 8 mM SBE-β-CD was added, respectively. In terms of solubility enhancement, SBE-β-CD is more efficient than β-CD in all three flavonoids.

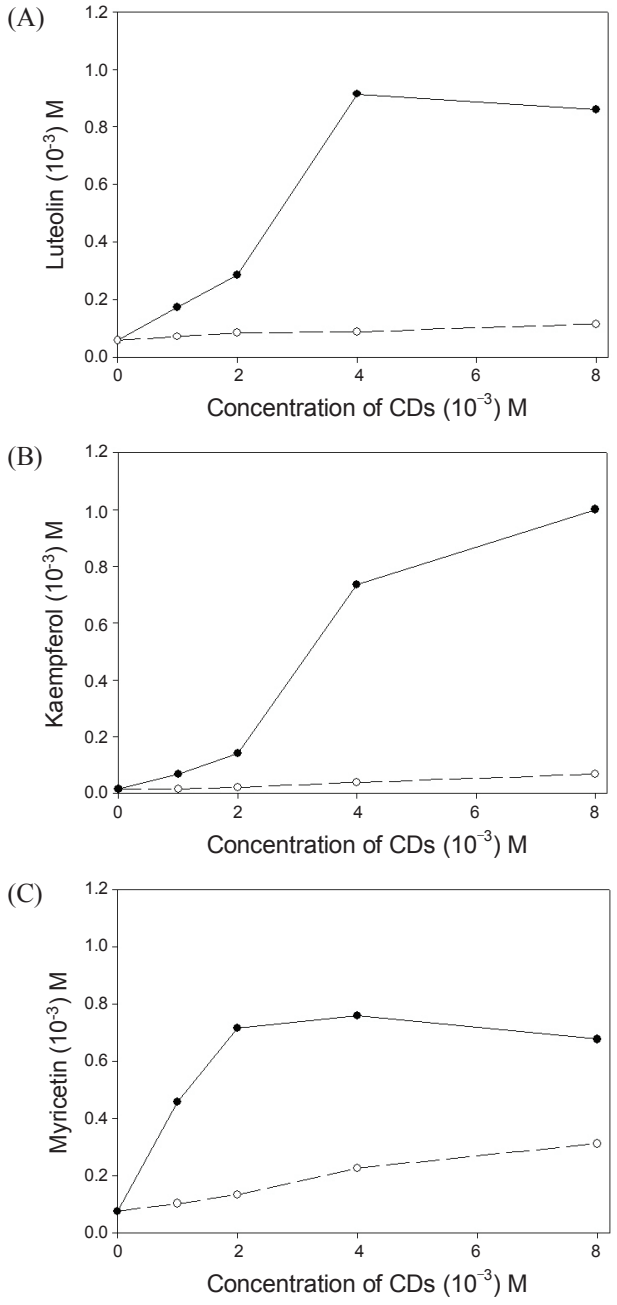

Figure 3. Phase solubility diagrams of flavonoid-CD complexes at 30 °C: (A) luteolin, (B) kaempferol and (C) myricetin with β-CD (○) and SBE-β-CD (●).

The phase solubility diagrams of flavonoids with β-CD and SBE-β-CD within the concentration range studied displayed a typical A_L type diagram consistent with a 1:1 molecular complex formation for two kinds of β-CDs.[18] The stability constant Ks were calculated from the straight-line portion of the phase solubility diagram and the stability constant equation after the following equation, Ks = slope/S_0 (1-slope). S_0 is the solubility of flavonoids in absence of CDs and slope means the slope of the phase-solubility diagram.[19] The values of stability constants for β-CD and SBE-β-CD complexes in water were calculated in Table 1. The order of stability constant with both CDs is kaempferol > myricetin > luteolin. The complexation of flavonoid with CDs is influenced by the structure of flavonoids. Luteolin-CD complexes show a less stability constant than other two flavonol-CD complexes. Kaempferol shows the highest stability constant with CDs than other two flavonoid complexes. Another flavonol, myricetin, has more hydroxyl groups than kaempferol, which might results in less affinity with the hydrophobic cavity of CDs than less hydroxyl group containing flavonoids.[14] Stability constants of all flavonoids-SBE-β-CD complexes were higher than those of β-CD complexes. The main driving forces of the CDs inclusion complex are in general hydrophobic interactions and hydrogen bond.[13] As sulfobutylether substituents of SBE-β-CD give more expanded hydrophobic cavities and more hydrogen bonding sites compared with native β-CD, SBE-β-CD may give the higher stability constant for its inclusion complexes.[20] Furthermore, SBE-β-CD maybe more useful alternative for the parenteral delivery of low water-soluble compounds[21,22] because it shows more aqueous solubility comparing with β-CD as well as it does not show any toxicity.

Antioxidant effects of luteolin, myricetin or kaempferol were also investigated based on the DPPH assay. DPPH is a stable free radical generating a deep violet solution in organic solvents.[19] Its progressive discoloration in the presence of luteolin, kaempferol or myricetin indicates the antioxidant activities of tested flavonoids. The rate of the DPPH-scavenging reaction was measured by observing the decrease in absorbance at 517 nm.[20] Figure 4 shows the consumption of the DPPH in different systems. The antioxidant activities of free flavonoids are different according to their structure. The order of antioxidant activity of free flavonoids is myricetin > luteolin > kaempferol (Figure 4)[6]. This order was not affected much even after the

Table 1. Stability constant (Ks) of flavonoid-CD complexes and solubility enhancement of flavonoids by CDs

	Stability constant Ks (M^{-1})[a]		Solubility Enhancement (times)[b]	
	β-CD	SBE-β-CD	β-CD	SBE-β-CD
Luteolin	128.05	2613.96	1.51	15.53
Kaempferol	402.50	9961.69	2.54	47.50
Myricetin	398.92	2948.24	3.00	10.07

[a]Stability constants of complexes are obtain from the phase solubility studies. [b][Solubilized flavonoids without CDs]/[Solubilized flavonoids with 4 mM CDs].

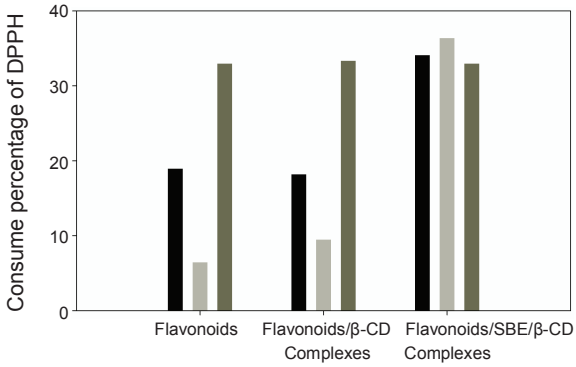

Figure 4. The consume percentage of DPPH in presence of luteolin (black bar), kaempferol (gray bar), and myricetin (dark gray bar) free and complexes forms.

complexation with β-CD. However, flavonoid-SBE-β-CD complexes change the order where the luteolin- or kaempferol-SBE-β-CD complexes show the higher antioxidant activities than free flavonoids or β-CD complexes do. In case of myricetin, no significant change was observed despite of the great solubility enhancement after the complexation with SBE-β-CD (Figure 4). Since the antioxidant activities of flavonoid-CD complexes may be determined by the molecular environment of specific antioxidant binding sites, the pyrogallol[6] moiety on the B ring of myricetin would be effectively protected by complexion with the CD cavities. Myricetin-CD complex showed no increase of antioxidant activity because its pyrogallol moiety hardly consumes DPPH than free myricetin. This result suggests the complexation of myricetin with CDs would occur around the pyrogallol moiety on the B ring of myricetin.

In conclusion, β-CD and SBE-β-CD functioned as a solubilizing agent against three flavonoids. SBE-β-CD is more efficient than native β-CD in solubility enhancement of tested flavonoids. All three tested flavonoids have antioxidant ability. Flavonoid-CD complex positively affected the antioxidant activity comparing with free flavonoids. Throughout this research, SBE-β-CD showed better complexation capacity for the solubility enhancement and bioavailability of tested flavonoids comparing with native β-CD.

Experimental Sections

Chemicals. Luteolin(3',4',5,7-Tetrahydroxylflavone), Kaempferol(3,5,7,4'-tetrahydroxyflavone), Myricetin(3,5,7,3', 4',5'-Hexahydroxyflavone) were purchased from Indofine chemical company (Hillsborough, USA). DPPH (2, 2-Diphenyl-1-pikryl-hydrazyl) and β-CD were purchased from Sigma Aldrich (St. Louis, MO, USA). SBE-β-CD (Total degree of substitution = 6-7; Captisol®) was purchased from Cydex, Inc. (Lenexa, USA).

Phase-solubility measurement. UV-vis absorption spectra were obtained with a spectrophotometer UV-vis spectrophotometry (UV 2450, Shimadzu Corporation). Prepare 1 mM flavonoid in 1 mL methanol solution and added to distilled water solution of each concentration cyclodextrins (0, 1, 2, 4, 8 mM) were prepared in 10 mL vial with magnetic stirring bar. The all vials were covered with aluminum foil and then flavonoids mixed with β-CD, SBE-β-CD solution during 24 hr at 30 °C, 250 rpm in dark. After the equilibrium, the mixture was evaporated and lyophilized. The samples were added 1 mL diluted water and filtered through 0.2 μm syringe filter.[21]

DPPH scavenging capacity assay. The antioxidant activity was measured, wherein the bleaching rate of a stable free radical, DPPH is monitored at a characteristic wavelength in the presence of the samples. In its radical form, DPPH absorbs at 517 nm,[19] but upon reduction by an antioxidant or a radical species its absorption decreases. A volume of 2 mL of 50 μM DPPH was used. DPPH is insoluble in aqueous solution the scavenging study was operated in mixture of methanol-water (20:80).[20] The reaction was started by addition of 20 μL of each free Flavonoids and Flavonoid complex samples, which used to the 4 mM cyclodextrin concentration from the phase-solubility studies. The decrease in absorbance at 517 nm was measured against a blank of pure methanol to estimate the radical scavenging capacity of each antioxidant sample. The results were expressed as percentage of DPPH eradication calculated according to the following equation: $AU = [1 - A_S/A_0] \times 100$,[22] where AU is radical-scavenging activity, the absorbance of sample is A_S and blank sample is A_0.

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