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Solubility Enhancement of Flavonols in the Inclusion Complex with Thioether-bridged Dimeric β-Cyclodextrins

Eunae Cho, Daham Jeong, Hyun-Dong Paik,† and Seunho Jung*

Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center (BMIC) & Institute for Ubiquitous Information Technology and Applications (CBRU), Konkuk University, Seoul 143-701, Korea
**E-mail: shjung@konkuk.ac.kr*
†Department of Food Science and Biotechnology of Animal Resources, Bio/Molecular Informatics Center (BMIC), Konkuk University, Seoul 143-701, Korea
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Dimeric β-cyclodextrin linked by a thioether bridge was synthesized from a reaction of mono-6-iodo-6-deoxy-β-cyclodextrin with sodium sulfide, and the structure was analyzed using nuclear magnetic resonance spectroscopy and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The effects of thioether-bridged dimeric β-CD on the aqueous solubility of flavonols (myricetin, quercetin, and kaempferol) were investigated by ultraviolet-visible spectroscopy. The aqueous solubility of myricetin, quercetin, and kaempferol were enhanced 33.6-, 12.4-, and 10.5-fold following the addition of 9 mM of thioether-bridged dimeric β-CD. In comparison, the aqueous solubility of myricetin, quercetin, and kaempferol were enhanced 5.4-, 3.3-, and 2.7-fold using the same concentration of monomeric β-cyclodextrin. Furthermore, the formation of flavonol/thioether-bridged dimeric β-CD inclusion complexes was confirmed with nuclear magnetic resonance, Fourier-transform infrared spectroscopy, differential scanning calorimetry, and scanning electron microscopy. The results showed that the nature of the complexes significantly differed from that of free flavonols. Herein, we suggest that the thioether-bridged dimeric β-CD can act as an effective complexing agent for flavonols.

Key Words : Thioether-bridged dimeric β-cyclodextrins, Flavonols, Complexation, Solubilization

Introduction

Flavonoids are a large class of secondary metabolites encompassing more than 10,000 structures. Among them, flavonols, which constitute a major group, have a 3-hydroxyflavone backbone and are present in vegetables and fruits, including citrus, red grapes, onions, and broccoli, as well as in nature in pine bark. They fulfill multiple functions in plants, including pigmentation in flowers, inhibition of reactive oxygen species generation, and protection from microbial and insect attacks.[1-4] Furthermore, they are beneficial to human health, as they possess several anticarcinogenic, antiallergic, and antiviral properties.[5-7] Indeed, there is a positive correlation between diets that are high in fruits and vegetables and a decreased risk of cancer. However, the potential clinical utility of flavonols is limited because of their low aqueous solubility. A plausible strategy to increase flavonol solubility is its complexation with cyclodextrins (CD), molecules capable of changing the physicochemical properties of flavonols, thus favoring their bioavailability.

Inclusion complexation occurs when electrostatic, van der Waals, and hydrophobic interactions and hydrogen bonding are cooperative. CDs are the most important and promising macrocyclic molecules, classified as α-, β-, and γ-CD and composed of six to eight glucose units connected through α-1,4 linkages. Owing to their bucket structure, they can capture suitable hydrophobic moieties into their hydrophobic cavity in an aqueous solution. Therefore, these species are widely used as separation reagents, enzyme mimics, sensors, and drug carriers.[8,9] Among them, β-CD is the most widely studied host molecule for a variety of aqueous species because of its availability and appropriate molecular size. However, because the inclusion by native CD is less efficient and less specific, β-CD has been extensively modified to improve its inclusion ability. Thus far, studies have reported that during the inclusion complexation between CD and flavonoids, the contact between water and the nonpolar region of flavonoids is reduced, thereby enabling flavonoids to be solubilized in aqueous media.[10-13]

Recently, dimeric β-CD species tethered by spacers of different sizes and shapes have attracted much attention as promising and versatile receptors for molecular recognition and as building blocks for functional materials.[14,15] It has been reported that the ability of dimeric β-CD species, especially those with two or more hydrophobic complexation sites, to complex with foreign molecules can be substantially enhanced owing to their stability, selectivity, and flexibility. Furthermore, cooperative complexations and a simple statistical advantage are expected owing to their dimeric structure. For example, certain linked dimeric β-CD species have been reported to selectively bind the hydrophobic side chain in polypeptides and modulate the polypeptide aggregation process.[16,17] In addition, dyes, drugs, and steroids have been studied for complexation and solubi-

lization by dimeric β-CD with disulfide, bipyridine, and diamine linkers.[18-21] Herein, we synthesized thioether-bridged dimeric β-cyclodextrins, and their structure was confirmed with nuclear magnetic resonance (NMR) spectroscopy and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The aqueous solubility of flavonols is enhanced by complexation with dimeric β-CD, to our knowledge, this is the first report of flavonoids/dimeric β-CD complexation. Complex formation was analyzed with NMR, Fourier transform infrared (FT-IR) spectroscopy, differential scanning calorimetry (DSC), and scanning electron microscopy (SEM).

Experimental

Chemicals. β-CD (Tokyo Chemical Industry Co., Ltd.) was recrystallized from distilled water and dried *in vacuo* for 12 h. *N,N*-Dimethylformamide (DMF) was obtained from Alfa Aesar, a Johnson Matthey Company. Kaempferol (> 97.0%), quercetin (> 98.0%), and myricetin (> 97.0%) were purchased from Sigma–Aldrich Chemicals Co. and Tokyo Chemical Industry Co. LTD.

Synthesis of Thioether-bridged Dimeric β-CD. Thioether-bridged dimeric β-CD was synthesized from mono-6-*O*-*p*-toluenesulfonyl-β-CD, which is the most important intermediate required for modifying one of the 6-hydroxy groups of β-CD into other functional groups on the primary side. The mono-6-*O*-*p*-toluenesulfonyl-β-CD (1.00 g, 0.78 mmol) was synthesized as described previously,[22] and dissolved in anhydrous DMF (40 mL). To this solution, sodium iodide (1.20 g, 8 mmol) was added under nitrogen with vigorous stirring for 5 h at 90 °C. Addition of acetone (25 mL) led to the formation of a precipitate. After washing, the acetone was removed under reduced pressure (rotary evaporator). The resulting solid was dissolved in deionized water (50 mL) and precipitated by the addition of copious amounts of acetone. The precipitate was filtered and dried in a desiccator. The resulting 6-iodo-6-deoxy-β-CD (0.88 g, 0.71 mmol) was dissolved in 8 mL of dry DMF, and made to react with sodium sulfide (27.64 mg, 0.35 mmol) for 15 h at 80 °C.[21] The reaction mixture was concentrated under reduced pressure and precipitated with 200 mL of acetone. The precipitates were collected on a glass filter and dried under reduced pressure. After these precipitates were dissolved in water, thioether-bridged dimeric β-CD were separated using a Bio-Gel P6 column. The dried product was afforded in 13.5% yield from starting β-CD.

Matrix-assisted Laser Desorption/Ionization-time of Flight Mass Spectrometry. The mass spectra of the synthesized thioether-bridged dimeric β-CD were acquired using a MALDI-TOF mass spectrometer (Voyager-DE^{TM} STR BioSpectrometry; PerSeptive Biosystems, Framingham, MA, USA) in the positive-ion mode by using 2,5-dihydroxybenzoic acid (DHB) as the matrix.

Phase-solubility Study. Kaempferol, quercetin, and myricetin were dissolved in 3 mM of methanol, and the methanolic solution was added to aqueous solutions of monomeric and dimeric β-CD (0.0–9.0 mM). The mixtures were magnetically stirred for 24 h at 25 °C and shielded from light to prevent degradation of the molecules. After equilibration, the methanol in the suspensions was evaporated using nitrogen gas before lyophilizing them. The lyophilized samples were dissolved in distilled water, and filtered with a 0.2-μm syringe filter (PTFE syringe filter; Whatman). The filtrates were diluted 10-fold before detection. The amount of dissolved genistein and daidzein was analyzed using UV-vis spectrophotometry (UV 2450; Shimadzu Corporation). The spectra were obtained from 220−400 nm, and the apparent stability constant (K_s) values of flavonols with monomeric and dimeric β-CD complexes were calculated on the basis of phase-solubility diagrams by using the following equation:

$$K_s\ (M^{-1}) = \text{slope}/S_0\ (1\text{-slope})$$

Where S_0 is the solubility of flavonols in absence of monomeric and dimeric β-CD and slope is the slope of the phase-solubility diagram.

Preparation of Complexes of Flavonols with Dimeric β-CD. Inclusion complexes of flavonols and dimeric β-CD were prepared using the suspension method. In a typical procedure, flavonols were dissolved in methanol and then added to an aqueous solution of an appropriate concentration of dimeric β-CD. The resulting mixture was stirred at 25 °C for 24 h. After equilibration, methanol was evaporated using nitrogen gas and lyophilized. The dissolved sample was lyophilized again to remove water.

Nuclear Magnetic Resonance Spectroscopy. For the NMR spectroscopic analysis, a Bruker Avance 500 spectrometer was used to record the ^{1}H–NMR, ^{13}C-NMR, and Heteronuclear Single Quantum Coherence (HSQC) spectra. NMR analyses were performed under deuterium oxide at room temperature.

Scanning Electron Microscopy. Samples were mounted onto stubs using double-sided adhesive tape and then made electrically conductive using a coating of a thin gold layer. The surface morphologies of the materials were examined under a scanning electron microscope (JSM 6380; Jeol, Tokyo, Japan). Images were taken at an accelerating voltage of 20 kV.

Differential Sacanning Calorimetry. The crystallinity of flavonols and their complexes was characterized by using a DSC 7020 instrument (SEICO INST.). A 2 mg sample was placed into sealed aluminum pans prior to heating under nitrogen (40 mL/min) at a scanning rate of 10 °C/min. The observations were recorded over the temperature range of 30–400 °C. An indium standard was used to calibrate the temperature scale.

Fourier-transform Infrared Spectroscopy. FT-IR spectra were obtained in potassium bromide matrix by using Bruker IFS-66 spectrometer (AMX, Germany). The spectra were recorded in the scanning range was 400–4000 cm^{-1}.

Results and Discussion

Molecules of dimeric β-CD tethered by thioether were

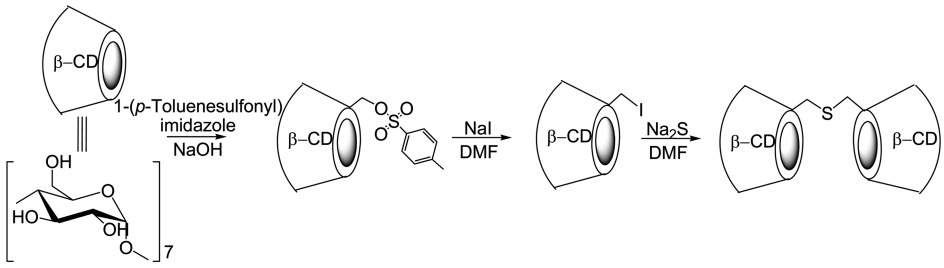

Figure 1. Synthetic scheme of thioether-bridged dimeric β-cyclodextrin.

prepared through monotosylation, iodation, and dimerization, as described in the experimental methods section (Figure 1). After purification on a Bio-gel P6 column, molecules of dimeric β-CD were obtained and their resulting structures were analyzed by NMR spectroscopy and MALDI TOF mass spectrometry. The pseudo-molecular ion peak at *m/z* = 2291.1 as [thioether-bridged dimeric β-CD + Na + 2H]$^+$ is shown in its MALDI-TOF mass spectrum. In its HSQC spectrum (Figure 2), compared to the C6 carbons (61.5 ppm) with the free OH group, the substituted C6' carbons shifted significantly upfield to 35.7 ppm, which correlated with the methylene germinal H6' resonances attached to the C6' carbons. The H1 signals of glucose residues showed a unique pattern resembling those of a mono substituted CD derivative, whereas H1 signals of monomeric β-CD appeared with doublet.[23] Additionally, a moderate upfield shift for H4' (3.5 ppm) and moderate downfield shifts for H5' (4.0 ppm) were correlated with C4' and C5' carbons, respectively. Other protons were also subjected to some shifts but their assignments were not attempted. These structural analyses indicate that thioether-bridged dimeric β-CD were successfully synthesized.

Since increasing the area of the hydrophobic recognition site of a host molecule strengthens the hydrophobic binding, double or triple recognition gives rise to a new strategy for the modeling of more sophisticated complexation. As one example of multiple recognition, 6-(4'-(toluidinyl)naphthalene)-2-sulfonate containing two hydrophobic recognition elements have shown the association constant with the linked dimeric β-CD ranging from 3.5 to 18.2 times greater than that with the corresponding β-CD monomer.[24] In this respect, bioactive flavonols which have two hydrophobic parts were considered as a possible guest for the synthesized dimeric β-CD with thioether bridge. Inclusion capacity of

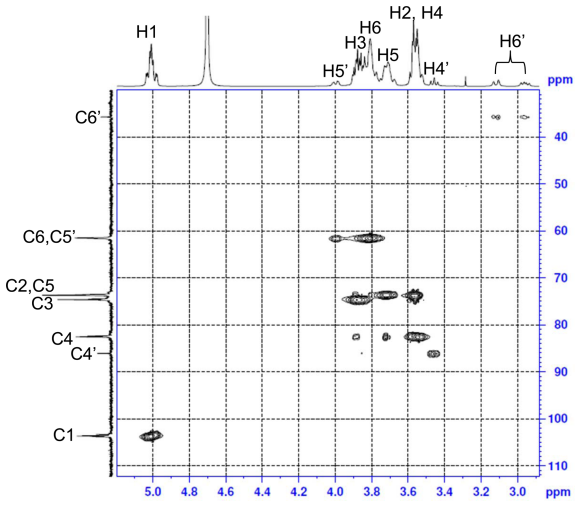

Figure 2. Heteronuclear single quantum coherence (HSQC) spectrum of thioether-bridged dimeric β-cyclodextrin.

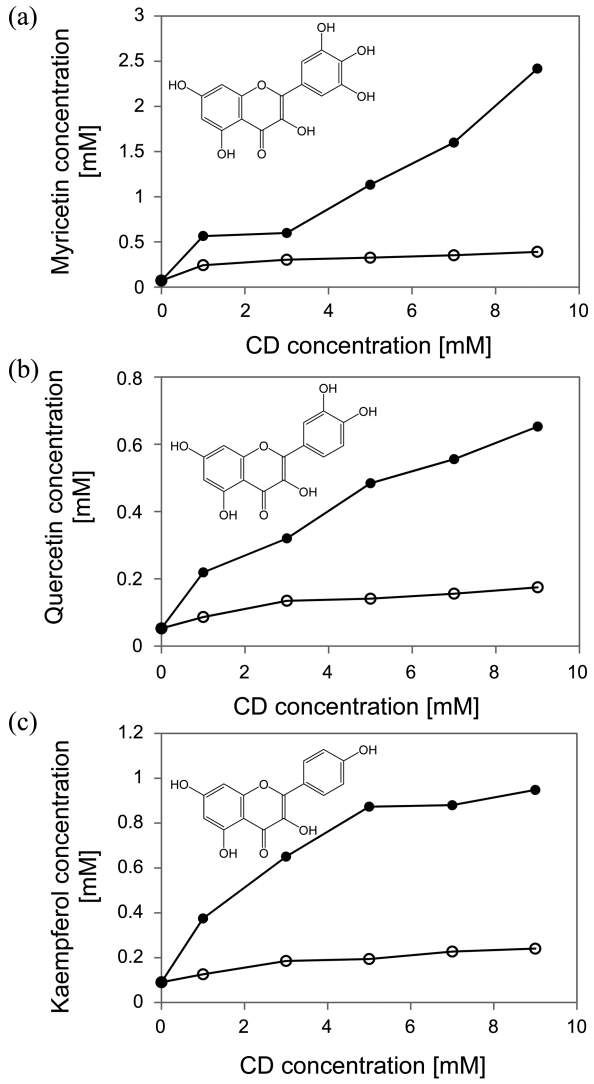

Figure 3. Phase solubility diagram of myricetin (a), quercetin (b), and kaempferol (c) with monomeric ○ and dimeric β-CD ●. Insets show the chemical structures of myricetin, quercetin, and kaempferol.

dimeric β-CD on flavonols was investigated by UV-visible spectroscopy, and the phase solubility diagram was obtained by measuring solubilized myricetin, quercetin, and kaempferol in the presence of increasing concentrations of dimeric β-CD (Figure 3). Myricetin, quercetin, and kaempferol have different numbers of hydroxyl groups in the B ring as shown in inset figures (Figure 3). The phase solubility diagram described by Higuchi and Connors is widely accepted for evaluating the effect of complexation on drug solubility.[25] For all the flavonol/dimeric β-CD systems, type-A_L diagrams were obtained, which indicated that the aqueous solubility of the flavonols was apparently increased by the presence of dimeric β-CD. Assuming a 1:1 stoichiometry of the complexes, K_s values were calculated from the straight-line portion of the phase-solubility diagram. In the case of kaempferol, because the absorbance value reached a plateau at 5 mM of dimeric β-CD (Figure 3(c)), the slope of the curve was evaluated at concentrations ranging from 0 to 5 mM.[26]

The stability constants of the various flavonols complexed with dimeric β-CD were 4315 M^{-1} for myricetin, 2233 M^{-1} for kaempferol, and 1497 M^{-1} for quercetin (Table 1). The calculated values are higher than those (594 M^{-1} for myricetin, 214 M^{-1} for kaempferol, and 289 M^{-1} for quercetin) obtained when the flavonols were complexed with monomeric β-CD. In both monomeric and dimeric β-CD, the stability constant of myricetin is higher than those of quercetin and kaempferol. This trend is influenced by a delicate difference of flavonol structures, where myricetin, quercetin, and kaempferol have three, two, and one hydroxyl groups in the B ring, respectively. Thus, considering the complexation model, the superior stability constant of myricetin can be derived from the effective complexation between B ring of myricetin and the inner cavity of β-CD. Furthermore, double recognition by the dimeric β-CD is supposed to enhance the efficiency for complexation with myricetin. In the case of a

Table 1. Stability constants for flavonols β-cyclodextrin (β-CD) and flavonols/thioether-bridged dimeric β-CD

Flavonols	β-CD K_s (M^{-1})	Thioether-bridged dimeric β-CD K_s (M^{-1})
Myricetin	594	4315
Quercetin	289	1497
Kaempferol	214	2233

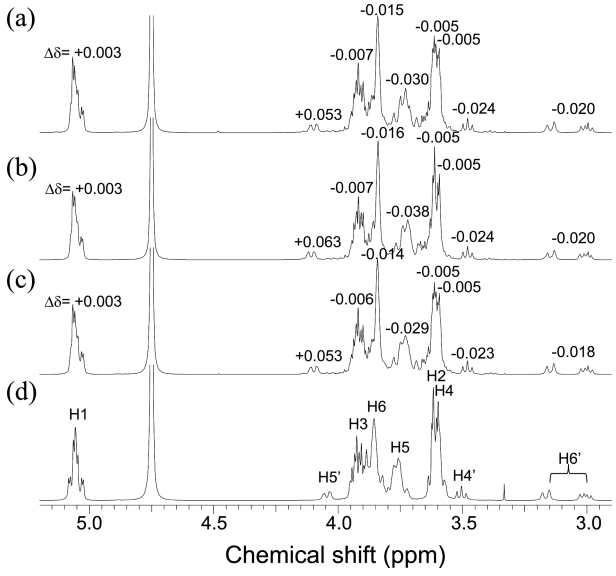

Figure 4. Partial ^{1}H-NMR spectra: inclusion complex with myricetin (a); inclusion complex with quercetin (b); inclusion complex with kaempferol (c); free thioether-bridged dimeric β-cyclodextrin (d). [flavonol] = 3 mM and [thioether-bridged dimeric β-cyclodextrin] = 7 mM. Spectra recorded in D_2O; signals referred to residual HDO.

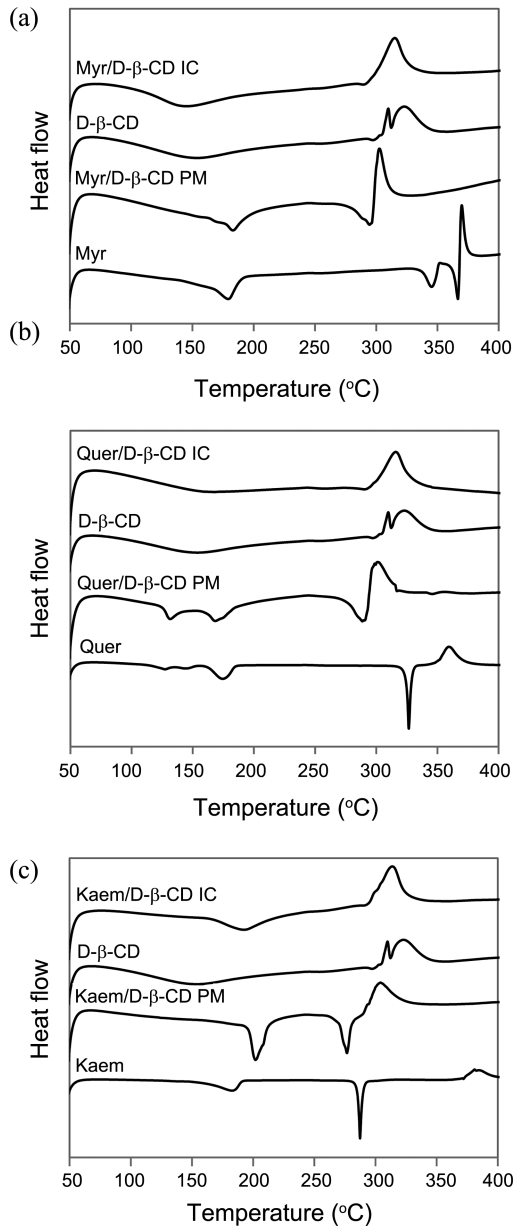

Figure 5. Differential scanning calorimetry (DSC) curves of flavonols/D-β-CD inclusion complex, D-β-CD, flavonols/D-β-CD physical mixture, and pure flavonols; Myricetin (a); quercetin (b); kaempferol (c). Myr: myricetin, Quer: quercetin, Kaem: kaempferol, D-β-CD: dimeric D-β-CD, IC: inclusion complex, PM: physical mixture.

guest dye molecule, methyl orange/ethylene diamine linked dimeric β-CD has also been reported to have the K_s value, 3160 M^{-1}, whereas K_s of methyl orange/the corresponding β-CD monomer is 520 M^{-1}.[19] As the final outcome, the aqueous solubility of myricetin, kaempferol, and quercetin in 9 mM dimeric β-CD solution increased by 33.6-, 12.4-, and 10.5-fold, respectively, whereas monomeric β-CD showed only a 5.4-, 3.3-, and 2.7-fold enhancement. Among flavonols, dimeric β-CD showed the best solubilizing efficiency for myricetin, which was mostly solubilized in the presence of 9 mM dimeric β-CD. These results suggest cooperative complexation of flavonols by dimeric β-CD because the difference in the solubilizing effect is more than a simple statistical advantage.

^{1}H-NMR spectroscopy is a suitable method for the evaluation of non–covalent interactions at the molecular level.[27] Signals of the flavonols were hardly detectable in the spectra of the complexes due to the extremely poor aqueous solubility and the presence of a great amount of dimeric β-CD.[28] Thus, the change of peaks corresponding to dimeric β-CD was analyzed, where the proton peaks of dimeric β-CD were shifted by the inclusion with myricetin, quercetin, and kaempferol (Figure 4). The assignments of the proton signals of dimeric β-CD are shown in Figure 4(d), and H5 located inside the torus of β-CD experienced the most affected shift. The chemical shift change (Δδ) is defined as the difference in chemical shift change, positive sign means a down-field shift and negative sign means an up-field shift. The up-field shift of H5 is undoubtedly attributed to the ring current effects generated by the circulating electrons of the aromatic guest, and the magnitude is similar to that of 3-hydroxyflavone interacted with β-CD monomer.[28] Although H1 and H3 were shifted less, peak shapes were clearly changed by the inclusion process. The unique down-field and up-field shifts were also observed in H5', H4' and H6' protons of thioether linked dimeric β-CD, and this might contribute favorably to the double recognition. These ^{1}H-NMR analyses provide the indication for the formation of the inclusion complex between dimeric β-CD and flavonols.

Thermal analysis methods, particularly DSC, are widely used in pharmaceutical fields, in analyses ranging from control of raw materials to stability and in pre-formulation studies for the development of new formulations. Figure 5 shows the DSC thermograms of the flavonol/dimeric β-CD inclusion complexes, dimeric β-CD, flavonol/dimeric physical mixtures, and free flavonols. The DSC curve of myricetin, quercetin, and kaempferol exhibited a sharp endothermic peak at 366.7 °C, 326.6 °C, and 287.5 °C, indicating their melting points. The observed melting point values are similar to their reported melting point values of 360 °C, 316 °C, and 277 °C, respectively. The physical mixtures of flavonols and dimeric β-CD showed the characteristic peak at 294.4 °C for myricetin, 288.6 °C for quercetin, and 276.8 °C for kaempferol; these lower temperature can be attributed to the chemical interaction between the flavonols and dimeric β-CD during the heating process inherent in DSC experiments. This phenomenon has been previously observed when a physical mixture of olanzapine and methyl β-CD was analyzed.[29] However, in the DSC curve of flavonol/dimeric β-CD inclusion complexes, the flavonol endothermic peak completely disappeared, and the characteristic exothermic peak of dimeric β-CD changed, suggesting a successful formation of an inclusion complex between the two compounds.

Morphological changes are frequently employed as a tool to evaluate the interaction between drugs and hosts.[30] In the present study, SEM analysis was performed to investigate the morphologies of flavonol/dimeric β-CD inclusion com-

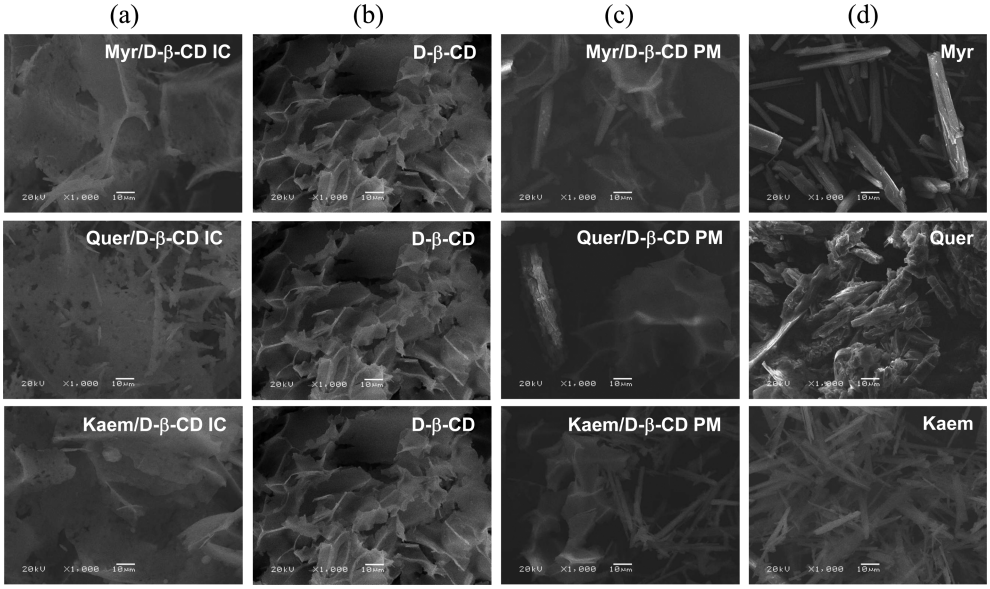

Figure 6. Scanning electron microscopy (SEM) images of flavonol/D-β-CD inclusion complexes (a), D-β-CD (b), flavonol/D-β-CD physical mixtures (c), and pure flavonols (d). Myr: myricetin, Quer: quercetin, Kaem: kaempferol, D-β-CD: dimeric β-CD, IC: inclusion complex, PM: physical mixture.

plexes, dimeric β-CD, flavonol/dimeric β-CD physical mixtures, and flavonols alone (Figure 6). Although this technique does not precisely confirm complex formation, it helps to confirm homogeneity as well as the approximate size of a single component. Myricetin and kaempferol show a smooth needle shape with lengths ranging from 30–50 mm, and quercetin shows an uneven rod shape of approximately 20 mm in length. The physical mixtures of flavonols and dimeric β-CD showed a combined morphology of regular shaped crystals and amorphous particles. After complexation, the original morphologies of flavonols and dimeric β-CD disappeared. Thus, the data obtained from SEM suggest the inclusion complex formation between flavonols and dimeric β-CD occurs in the solid state.

FT-IR spectroscopy was used to analyze vibrational changes upon the inclusion of flavonols with dimeric β-CD.

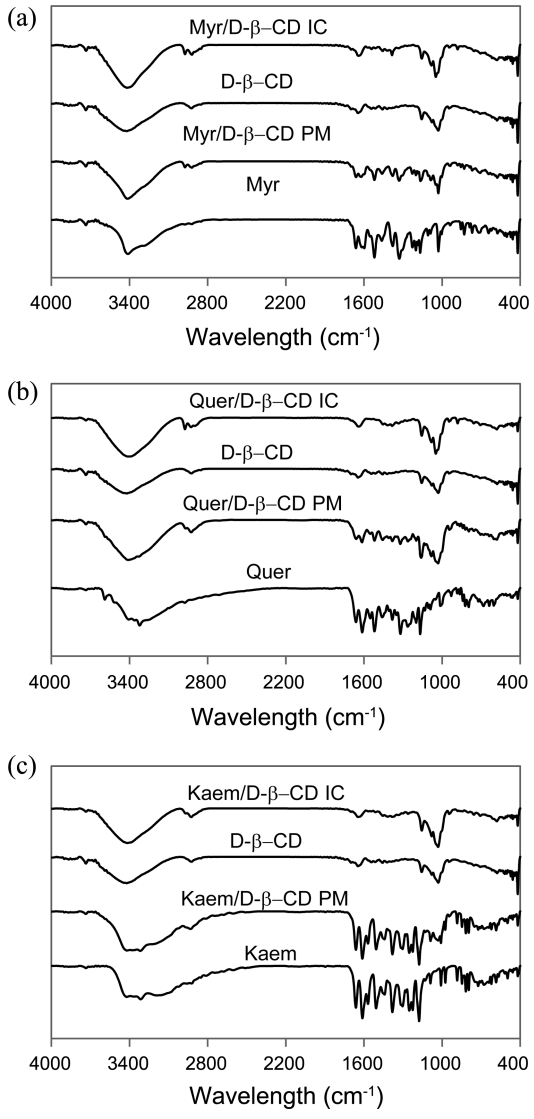

Figure 7. FT-IR spectra of flavonols/D-β-CD inclusion complex, D-β-CD, flavonols/D-β-CD physical mixture, and pure flavonols; Myricetin (a); quercetin (b); kaempferol (c). Myr: myricetin, Quer: quercetin, Kaem: kaempferol, D-β-CD: dimeric D-β-CD, IC: inclusion complex, PM: physical mixture.

Figure 7 shows the FT-IR spectra of flavonol/dimeric β-CD inclusion complexes, dimeric β-CD, flavonol/dimeric β-CD physical mixtures, and flavonols alone. For myricetin, the band at 3417 cm^{-1} was assigned to a free –OH bond vibration, and the bands at 1660 cm^{-1} and 1621 cm^{-1} were assigned to the stretching vibration of the C=O group that appeared as a very strong doublet. The bands at 1520 cm^{-1} and 1461 cm^{-1} were assigned to an aromatic C=C stretch and those at 1329 cm^{-1} and 1166 cm^{-1} were assigned to the C–O–C vibration (Figure 7(a)). For quercetin, the band at 3322 cm^{-1} was assigned to a free –OH bond vibration and those at 1661 cm^{-1} and 1614 cm^{-1} bands were assigned to the stretching vibration of the C=O group that again appeared as a very strong doublet. Additionally, the band at 1507 cm^{-1} was assigned to an aromatic group and those 1320 cm^{-1} and 1168 cm^{-1} bands were assigned to the C–O–C vibration. (Figure 7(b)). Kaempferol showed a band at 3314 cm^{-1} assigned to a free –OH bond vibration, and the bands at 1662 cm^{-1} and 1612 cm^{-1} were assigned to the stretching vibration of the C=O group. Kaempferol also showed a band at 1507 cm^{-1} was assigned to an aromatic group, and those at 1304 cm^{-1} and 1176 cm^{-1} bands were assigned to the C–O–C vibration (Figure 7(c)). The dimeric β-CD spectrum displayed an intense band at 3421 cm^{-1}, indicative of the vibration of hydrogen–bonded OH groups; a band at 2924 cm^{-1}, indicative of the C-H stretch; a band at 1157 cm^{-1}, indicative of the C-O stretch; and a peak at 1029 cm^{-1}, indicative of the C-O-C vibration. In the case of physical mixtures, all characteristic bands of flavonols and dimeric β-CD were observed; however, the spectrum of its inclusion complex showed disappearance of typical flavonol peaks from 1656 to 1670 cm^{-1} and from 1598 to 1610 cm^{-1}. On comparing the myricetin/dimeric β-CD complexes with dimeric β-CD, some peaks, such as those attributed to –OH bond vibration, C-H stretch, and C-O-C vibration, shifted to 3419, 2920, and 1048 cm^{-1}, respectively, indicating that interactions between myricetin and dimeric β-CD had occurred. Similar changes were also observed in quercetin/dimeric β-CD complexes and kaempferol/dimeric β-CD complexes. From the solid-state characterization, we deduced that the results of the FT-IR spectroscopy confirmed not only an interaction but also the inclusion of flavonols with dimeric β-CD.

Conclusion

In conclusion, a thioether bridged dimeric β-CD was synthesized and used for the inclusion complexation of flavonols. The aqueous solubility of myricetin, quercetin, and kaempferol was enhanced by 33.6-, 12.4-, and 10.5-fold after complex formation with 9 mM dimeric β-CD. The solubilizing effect of dimeric β-CD was clearly distinctive from that of monomeric β-CD owing to the cooperative complexation ability of dimeric β-CD. The present thioether-bridged β-CD dimer would approximately match the separation of two hydrophobic aromatic components of flavonols, and enhance its complexation. The formation of the complex was confirmed with NMR, FT-IR spectroscopy,

DSC, and SEM. Thus, dimeric β-CD holds great promise for use as an effective complexation agent for flavonols. The complexing data can be used to compare flavonols and complexed flavonols, thereby facilitating the development of viable, safe, and effective formulations with increased solubility and stability. These characteristics are essential for good bioavailability of flavonols, which can in turn improve the clinical application of flavonols.

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