Carbohydrate Polymers 97 (2013) 196–202

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Complexation of fisetin with novel cyclosophoroase dimer to improve solubility and bioavailability

Daham Jeong[a], Jae Min Choi[a], Youngjin Choi[b], Karpjoo Jeong[c], Eunae Cho[a], Seunho Jung[a,*]

[a] Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center & Institute for Ubiquitous Information Technology and Applications (CBRU), Konkuk University, Seoul 143-701, South Korea
[b] BioChip Research Center, Hoseo University, Asan 336-795, South Korea
[c] Department of Advanced Technology Fusion, Institute for Ubiquitous Information Technology and Applications (CBRU), Konkuk University, Seoul 143-701, South Korea

ARTICLE INFO

Article history:
Received 26 March 2013
Accepted 17 April 2013
Available online 2 May 2013

Keywords:
Cyclosophoraoses
Cationic cyclosophoraose dimer
Rhizobium leguminosarum
Fisetin
β-Cyclodextrin
Solubility

ABSTRACT

Rhizobium species produce cyclosophoraose (Cys), which is an unbranched cyclic β-(1,2)-glucan. We synthesized novel cationic cyclosophoraose dimer (Cys dimer) and its structure was confirmed *via* NMR spectroscopy and MALDI-TOF mass spectrometry analysis. In this study, we investigated the complexation of hardly soluble drug fisetin (3,3′,4′,7-tetrahydroxyflavone) with Cys dimer to improve the solubility of fisetin, and its solubility was increased up to 6.5-fold. The solubility of fisetin with Cys dimer showed 2.4-fold better than with β-cyclodextrin. The fisetin–Cys dimer complex was characterized by using, phase solubility diagram, 2D NMR, FT-IR spectroscopy, SEM, DSC analysis and molecular modeling. Through the molecular docking simulations, complexation ability of fisetin with host molecules were in the following order: Cys dimer > Cys monomer > β-CD. The fisetin–Cys dimer complex showed also higher cytotoxicity to HeLa cells than free fisetin, indicating that the Cys dimer to improve bioavailability of fisetin.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Cyclosophoraose (Cys), an exopolysaccharide produced by many species of the *Rhizobiaceae* family, is composed of unbranched cyclic oligosaccharides joined by glucose units through β-(1,2)-linkages (Abe, Amemura, & Higashi, 1982). It occurs in various sizes and exists in a neutral or anionic form due to some substituents (Amemura, Hisamatsu, Mitani, & Harada, 1983; Spaink, 1992). The exact biological function of Cys is not well characterized; however, Cys is known to play a critical role in bacterial nodule invasion and induction of the crown gall tumor (Dylan et al., 1986; Geremia et al., 1987). The Cys found in *Rhizobium leguminosarum* VF-39 contains rings with degrees of polymerization that range from 17 to 23 and therefore varies in size. In general, Cys is found in the periplasmic space of the bacterial cell and can be excreted into the extracellular space (Abe et al., 1982; Amemura et al., 1983). Previous studies have shown that Cys possesses the ability to form complexes with hydrophobic guest molecules including fluorescein, indomethacin, naproxen, paclitaxel, and vitamins (Koizumi et al., 1984; Kwon et al., 2012; Okada, Horiyama, & Koizumi, 1986). Furthermore, the applications of Cys in biotechnology can be broadened by modifying various substituents such as carboxymethyl (Lee, Park, Seo, Choi, & Jung, 2004), sulfonyl (Park, Lee, Kang, Jung, & Jung, 2004), and succinyl groups (Kwon and Jung, 2011).

Fisetin (3,3′,4′,7-tetrahydroxyflavone) produced from various vegetables and fruits such as cucumber, onion, persimmon, strawberry, and apple exhibit various biological activities including antioxidant, anti-inflammatory, anti-proliferative, and proapoptotic effects (Arai et al., 2000; Kimira, Arai, Shimoi, & Watanabe, 1998). The biological properties of fisetin enable it to suppress the proliferation of human cervical cancer HeLa cells (Ying et al., 2012). Regardless of the potential applications of fisetin, it is not used *in vivo* because of its poor water solubility (<1 mg/mL; Guzzo et al., 2006). Therefore, nanoemulsion formulation, complex formation, and molecular carrier characterization of fisetin have been previously investigated to improve its solubility and bioavailability (Chien, Shen, Huang, Ko, & Shih, 2010; Ragelle et al., 2012).

To improve complexation of hydrophobic drugs, oligomerization of cationic Cys is important because Cys oligomers can solubilize poorly soluble drugs more effectively than parent Cys can

* Corresponding author at: Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center & Institute for Ubiquitous Information Technology and Applications (CBRU), Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, South Korea. Tel.: +82 2 450 3520; fax: +82 2 452 3611.
E-mail address: shjung@konkuk.ac.kr (S. Jung).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.04.066

(Jeong, Piao, Kwon, & Jung, 2012). The cavities in Cys are narrower than those expected from sizes of their bulky rings (Choi, Yang, Kim, & Jung, 2000). Therefore, the Cys oligomer can form complexes with insoluble drugs through the interaction of 2 adjacent Cys moieties (Harada, Furue, & Nozakura, 1980). Moreover, the solubilized complex can be effectively delivered to cells because of the high affinity of the interior cations toward the anionic phospholipids in the cell membrane (Vaara, 1992).

In the present study, a cyclosophoraose dimer (Cys dimer) containing quaternary ammonium groups was synthesized by crosslinking epichlorohydrin and choline chloride. The synthesized Cys dimer was isolated using Bio-gel P6 (Bio-Rad, USA) and confirmed *via* nuclear magnetic resonance (NMR) spectroscopy and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Here, we first prepared the complex of insoluble fisetin by using the Cys dimer as a solubilizer. Formation of the fisetin–Cys dimer complex was verified through 2D NMR spectroscopy, Fourier transform infrared (FT-IR) spectroscopic, differential scanning calorimetry (DSC), scanning electron microscopy (SEM) analyses and molecular modeling. Furthermore, the improvement in the solubility of the complex was assessed *via* a phase solubility study, and the enhanced bioavailability of the complex was analyzed by evaluating the cytotoxicity against HeLa cells.

2. Materials and methods

2.1. Materials

β-Cyclodextrin (β-CD) and fisetin (>98% purity) were purchased from Sigma–Aldrich Chemicals Co. (St. Louis, MO, USA). D_2O (99.9% at D) and dimethyl sulfoxide-d6 (D, 99.9%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA); these reagents were used without further purification. The isolation and purification of Cys from *R. leguminosarum biovar* VF-39 were carried out as described in previous reports (Jeon, Kwon, Cho, & Jung, 2010; Kwon et al., 2012).

2.2. Preparation of Cys dimer

Cys dimer was prepared by crosslinking epichlorohydrin and choline chloride (Jeong et al., 2012; Li, Xiao, Li, & Zhong, 2004). A mixture comprising 2.83 g of Cys (870.7 μmol) in 10 mL of NaOH (5%, w/v) solution was mechanically stirred overnight at 25 °C. Choline chloride 349 mg (2.5 mmol) was then added into the Cys-solution rapidly, and then 3.47 g (37.5 mmol) of epichlorohydrin was added drop-wise over a period 60 min at room temperature. Next, the mixture was heated to 60 °C. During polymerization, the temperature was maintained at 60 °C and the mixture was stirred continuously at 600 rpm. After 4 h, the polymerization was stopped by neutralization with an aqueous hydrochloride acid solution (3 N). The reaction mixture was then separated using Bio-Gel P-6.

2.3. Phase solubility analysis and continuous variation method

Phase solubility studies were performed according to the method reported by Higuchi and Connors (Higuchi and Connors, 1965). Due to the low water solubility of fisetin, it was dissolved in methanol. An 100 μL of the methanolic fisetin solution (30 mM) was added to aqueous solutions (1 mL) containing different concentrations of Cys dimer (0, 0.5, 1.0, 1.5, 2.0, and 2.5 mM). The suspensions were magnetically stirred at 25 °C for 24 h, shielded from light to prevent degradation of the molecules. After equilibrium was reached, methanol was evaporated using N_2 gas, and the mixture was lyophilized. The lyophilized sample was dissolved in water and filtered with a PVDF 0.2 μm filter (Whatman). An aliquot from each vial was analyzed using a spectrophotometer (UV2450, Shimadzu Corporation) at a wavelength of 380 nm to determine the dissolved fisetin concentration. The concentrations of fisetin and Cys dimer were plotted using the obtained data. Using the Higuchi and Connor equation, we calculated the equilibrium constant for complex formation from the linear portion of the solubility diagram.

$$K_c = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (1)$$

The stoichiometry of the complex was determined using the continuous variation method (Job's plot; Job, 1928). The sum of the concentrations of both components was kept constant ([Cys dimer] + [fisetin] = 1.0 mM) and the molar fraction of (r = [Cya dimer]/[{Cys dimer} + {fisetin}]) was varied from 0.0 to 1.0. The stoichiometric ratio was obtained by plotting $\Delta\text{Abs} \cdot r$ (where ΔAbs is the difference in fisetin absorbance with and without Cys dimer) and by finding the r value corresponding to the extreme of this dependence.

2.4. Two-dimensional nuclear overhauser effect spectroscopy (NOESY)

After acquiring equilibration of fisetin and Cys dimer with an equimolar ratio in water at 25 °C, the resulting complex was collected and lyophilized. The samples were lyophilized and dissolved in 60% deuterated water (D_2O, 99.96%) and 40% dimethyl sulfoxide-d6 (D, 99.9%). The complex was assessed by performing NOESY experiment by using A Bruker 500 MHz spectrometer (AMX, Germany). The NOESY data were acquired with a spin-lock mixing time of 600 ms, the time domain data was zero filled to 2048 points in F2 and 256 points in F1.

2.5. FT-IR spectroscopic analysis

The IR spectra of fisetin, Cys dimer, physical mixture and the fisetin–Cys dimer complex were recorded with a Bruker IFS-66/Spectrometer in the region 2000–500 cm^{-1}. The fisetin and Cys dimer were co-grounded as equimolar ratios for physical mixture. Fisetin–Cys dimer complex was prepared equimolar ratios (1:1) by the procedure described phase solubility analysis. Potassium bromide pellets were used for all samples.

2.6. Differential scanning calorimetry (DSC)

Differential scanning calorimetry of fisetin, Cys dimer, physical and the fisetin–Cys dimer complex was performed using a differential scanning calorimeter DSC 7020 (SEICO INST, JPN). The DSC analysis was calibrated with indium and performed at a temperature-scanning speed of 10 °C min^{-1} in the temperature range of 50–400 °C.

2.7. Scanning electron microscopy (SEM) analysis

JSM-6380 (JEOL, JPN) scanning electron microscope was used to acquire the SEM images. Double-sided adhesive carbon tape was used to fix the powder samples on a brass stub. The powders were coated on the surface in a thin layer at 30 W for 30 s in a vacuum for making them electrically conductive. The images were photographed at an excitation of 1 kV.

2.8. Molecular modeling process

Structural model for the Cys dimer was constructed using MacroModel 9.9 product in Schrodinger suite 2012. At first,

monomeric Cys (DP = 21) structure was chemically modified by builder module of Maestro 9.3.5. Initial structure of Cys monomer was energy-minimized and subjected to macrocyclic conformational sampling module in the MacroModel. An appropriate model for the Cys monomer was calculated with OPLS2005 force field under large-scale low-frequency mode. Electrostatics was treated with GB/SA implicit solvation model and 10 kcal/mol of energy-window value was applied to the course of simulations. Eigenvectors were determined for every new global minimum of the Cys monomer. In total, 500 cycles of each simulation and conformational sampling was performed at high temperature (T = 1000 K). Resultant Cys monomer structure was duplicated, and combined by bond connection to build Cys dimer. The structure of Cys dimer was refined using Conformational Search module in the MacroModel. Final structural model of Cys dimer was obtained by this conformational sampling under mixed torsional/large-scale low-frequency mode with maximum 10,000 iterations.

Flexible-ligand docking jobs of fisetin upon each host molecule were performed by Glide 5.8 product (Friesner et al., 2004) in the Schrodinger software. The starting structure of fisetin was prepared from builder tool of the Maestro interface. Rectangular grid box was defined for each Cys monomer, Cys dimer, and β-CD using Receptor Grid Generation tool in the Glide. The Glide docking score was obtained using extra precision *XP* mode with Glide XP 5.0 scoring function which is suitable for accurate prediction. In this docking mode, 0.5 kcal/mol of energy window and distance-dependent dielectric constant (ε = 1) was applied to pose sampling process.

2.9. Cell culture

The human cervical adenocarcinoma cell line, HeLa, was purchased from a Korean Cell Line Bank (Seoul, Korea). The cells were maintained in Eagle's minimum essential medium (MEM, WelGENE Inc., Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS, WelGENE Inc., Daegu, Korea), 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C in a humidified incubator at 5% CO_2.

2.10. In vitro cytotoxicity assay

WST-1 (4-[3-{4-iodophenyl}-2-{4-nitrophenyl}-2H-5-tetrazolio]-1.3-benzene disulfonate) test enabled cytotoxicity assessment (Hilmi et al., 2003). Cells were grown in 96-well plates at a concentration of 5×10^3 cells/well for 24 h, and then treated with 0.1% dimethyl sulfoxide (DMSO) or fisetin at one of the various concentrations tested (0, 10, 20, 40, 60, 80, and 100 μM) and a Cys dimer complex with a molar ratio of 1:1 (equilibrium time of 24 h and not filtered). After a 24 h incubation, each well was treated with WST-1 and incubated for additional 4 h. The number of viable cells was directly proportional to the production of formazan after solubilization. The color intensity was assessed at a wavelength of 450 nm in a SpectraMax 190 microplate reader (Molecular Devices, Corp., CA, USA). The experiments were performed in triplicate.

3. Results and discussion

3.1. Structural analysis of Cys dimer

Cys dimer was synthesized through a one-step procedure by using epichlorohydrin and choline chloride. A proposed structure of the Cys dimer is shown in Fig. 1a, and Cys can be derived from 2,3-dihydroxypropoxy and choline-linked 2-hydroxypropoxy before dimerization. The dimerization was confirmed through ^{1}H NMR spectra as described previously (Jeong et al., 2012; Li et al., 2004). In addition, MALDI-TOF mass spectrometry was used to verify the number-average molecular weight of the Cys dimer. The number-average molecular mass of Cys dimer was determined to be 7334 Da, which was approximately twice the number-average molecular mass (3250 Da) of native Cys (Table 1). Phenol–sulfuric acid assay (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956) was used to confirm dimerization and the data revealed that Cys dimer and Cys had 39.78 and 20.6 glucose units, respectively (Table 1). The determined glucose unit values are within the expected range because Cys has 17–23 glucose residues. Thus, dimerization of Cys was confirmed. In addition, choline moieties containing quaternary ammonium groups were added to the Cys dimer by utilizing its cationic property (Fig. 1a). The ionic derivatives have been shown to form stronger complexes with drugs or chemicals that carry the opposite charge (Xiao & Cezar, 2005). In this respect, cationic Cys dimer has therapeutic potential including drug and gene delivery (Samal et al., 2012). Furthermore, an array of guest molecules can be added by altering the cationic charge and 3-dimensional conformation of the Cys dimer.

Table 1
Glucose unit, numbered average molecular weight (M_n) and stability constants, and docking information of host molecules and fisetin.

	Host molecules		
	β-CD	Cys	Cys dimer
Glucose unit[a]	6.8	20.6	39.78
M_n (Da)	1135	3250	7334
Stability constant $K_{1:1}$ (M^{-1})	896.68	–	2188.18
Glide docking score	−2.783	−3.552[b]	−4.875
Number of close contacts	136	115[b]	151

[a] Glucose unit was determined by the phenol–sulfuric acid method with D-glucose as standard.
[b] The values of Cys monomer which is Cys modified with three 2,3-dihydroxy propoxy and one choline-linked 2-hydroxypropoxy residues. The Cys monomer is estimated based on the numbered average molecular weight of Cys dimer.

3.2. Phase solubility studies and continuous variation plot

Phase solubility diagrams of fisetin by β-CD, Cys dimer, and Cys were obtained using the UV spectra of different sample concentrations (Fig. 2a). The plot of fisetin and β-CD appeared to be A_L type, showing that the concentration of β-CD was directly proportional to the solubility of fisetin (Del Valle, 2004). The linear graph derived from fisetin and Cys dimer suggested a 1:1 molecular association. The solubilizing effect of the Cys dimer was approximately 2.4 times that of β-CD. On the other hand, the concentration of Cys did not show any effect on the solubility of fisetin because Cys does not form a complex with fisetin. The respective stability constants, K, were determined using Eq. (1) and are summarized in Table 1.

The stoichiometry of the complex was assessed *via* Job's method. As seen in Fig. 2b, the highest molar fraction was observed at 0.5, indicating that Cys dimer and fisetin formed a complex in 1:1 ratio, which was similar to the ratio obtained using the phase solubility diagram.

3.3. Two-dimensional nuclear overhauser effect spectroscopy (NOESY) analysis

NMR spectroscopic analysis is the ideal way to confirm complex formation (Misiuk & Zalewska, 2009). Two-dimensional NOESY analysis provides important information about the spatial proximity between host and guest atoms by determining the intermolecular dipolar cross-correlations (Yang et al., 2011). The ^{1}H NMR spectra showed that the chemical shift of the proton in fisetin remained unchanged before and after complex formation and showed a decline in split patterns (data not shown). A correlation between the protons of fisetin and those of Cys dimer was determined by using the NOESY spectrum (Fig. 3). The proton H-5

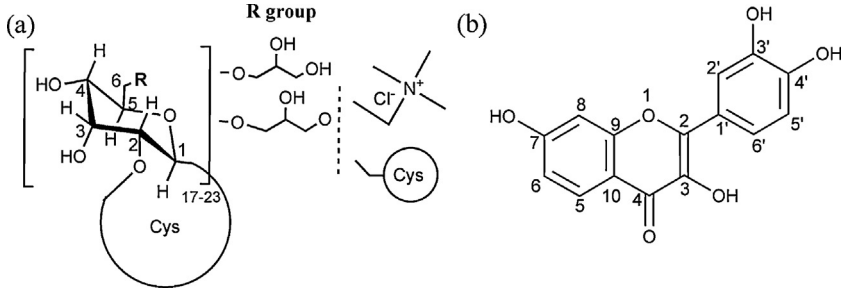

Fig. 1. Chemical structure of the cyclosophoraose dimer (a) and fisetin (b).

of fisetin was correlated with H-1–6 of Cys. Additionally, protons H-7, H-8, H-2′, H-5′, and H-6′ of fisetin were correlated with H-2–6 of Cys dimer. At 0.4 nm, the proton of fisetin and that of the Cys dimer appeared to be adjacent, validating that the complex was formed by the Cys dimer wrapping around fisetin instead of binding at a specific location (Schneider, Hacket, Rüdiger, & Ikeda, 1998).

3.4. FT-IR spectroscopic analysis

The variation in the shape, shift, and intensity of the FT-IR absorption peaks of the guest or host can provide sufficient information to confirm formation of a complex (Szente, 1996). The FT-IR spectra of fisetin, Cys dimer, and the fisetin–Cys dimer complex (c) can be seen in Fig. 4a. The FT-IR absorption bands for Cys dimer are 1631 cm^{-1} (O—H bending), 1407 cm^{-1} (O—H deformation), and 1074 cm^{-1} (C—O—C stretching). Fisetin is characterized by absorption bands appearing at 1618 cm^{-1} (C=O stretching, ●), 1570 cm^{-1} (C—C stretching, ▲), 1504 cm^{-1} (C—O stretching, Δ) and 1280 cm^{-1} (C—O—H bending, ■). These characteristic bands were reduced their intensities at the same position of physical mixture spectrum. However, these bands disappear after complex formation with Cys dimer. These results indicate that the environment of drugs in the complex was changed because the Cys dimer wraps around the aromatic rings of fisetin, which was in accordance with the NOESY result.

3.5. Differential scanning calorimetry (DSC)

The DSC spectrum provides information about the solid-state interaction between fisetin and the Cys dimer. When guest molecules were embedded into host molecules, the melting, boiling, or sublimating temperatures of the guest molecules generally shift to different temperatures or disappear (Giordano, Novak, & Moyano, 2001). Fisetin melts with an endothermic peak at 346.8 °C, but the endothermic peak disappears in the complex with Cys dimer (Fig. 4b). This result indicates that the peak of fisetin is not detected when the crystalline-active fisetin molecule interacts with the Cys dimer through various combinations of hydrogen bonds or van der Waals force (Wen, Liu, Yuan, Ma, & Zhu, 2010).

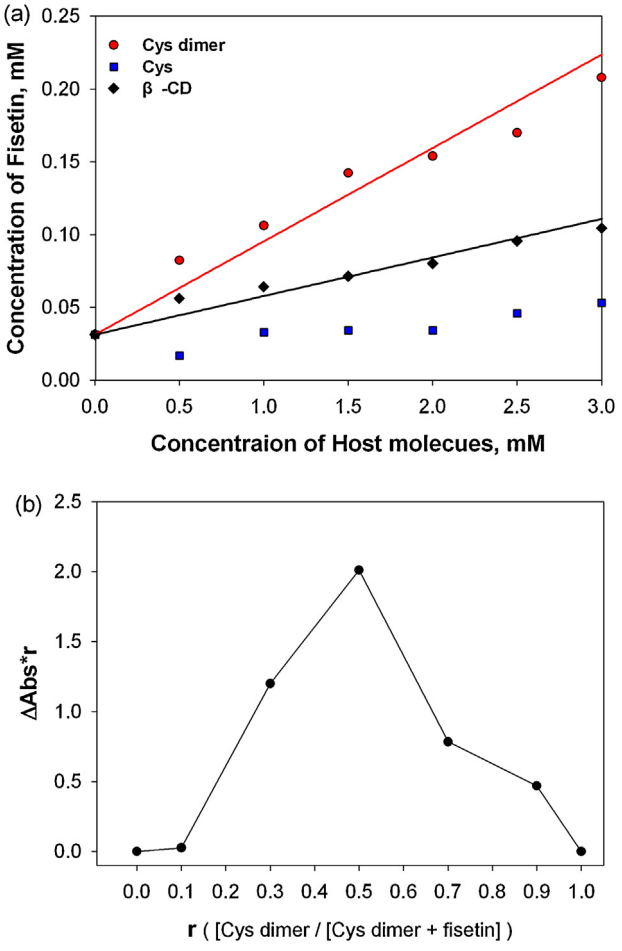

Fig. 2. (a) Phase solubility diagram of fisetin with Cys (■), Cys dimer (●) and β-CD (♦) and (b) Job's plot for fisetin and Cys dimer.

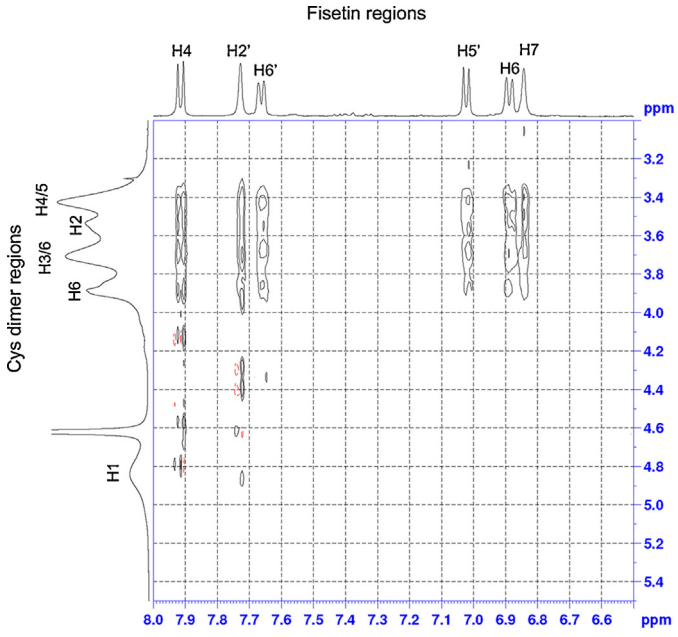

Fig. 3. Partial nuclear overhauser effect spectroscopy spectrum for the fisetin–Cys dimer complex.

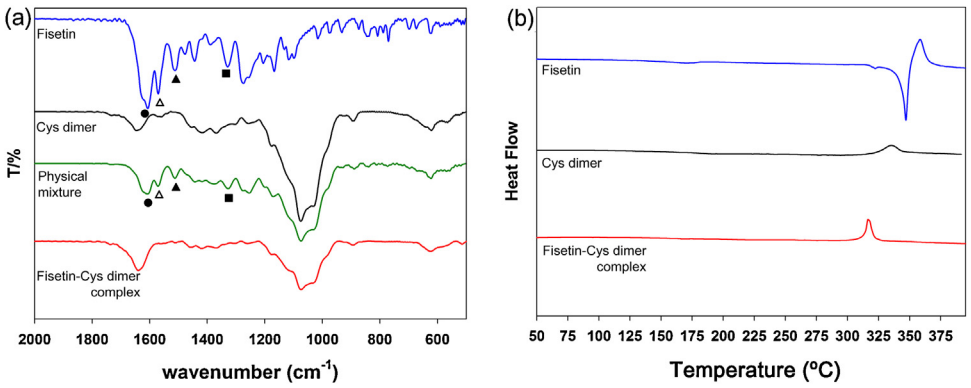

Fig. 4. FT-IR spectra (a) and DSC spectrum (b) of fisetin, Cys dimer, physical mixture and fisetin–Cys dimer complex.

3.6. Scanning electron microscopy (SEM) analysis

Morphological changes in the structure of the drug after complex formation were assessed using SEM analysis. The structural changes in the drug also indicate a change in its solubility (Lipinski, 2000). Scanning electron micrographs of fisetin (a), Cys dimer (b), physical mixture and the fisetin–Cys dimer complex (c) are displayed in Fig. 5. The fisetin structure prior to complex formation resembled varying sizes of stone-like figures, where as the Cys dimer consisted of rather planar sheets. The both shape and structure remained in the physical mixture (Fig. 5c). After complex formation, however, the stone-like figure of fisetin disappeared and only planar pieces remained. Thus, we concluded that fisetin was completely included in the Cys dimer and a solid Cys dimer complex was formed successfully.

3.7. Structural model of Cys dimer–fisetin complex

The characteristics of binding mode of complex between fisetin and Cys dimer were traced with molecular simulations by using Glide docking suite. The docking scores of fisetin with β-CD, Cys monomer, and Cys dimer were −2.783, −3.552, and −4.875, respectively. These results suggest Cys dimer is suitable complexation molecule for the fisetin compared to β-CD or Cys monomer. To address molecular details of the fisetin–Cys dimer complex, we have analyzed binding mode of each docked pose.

The fisetin made a typical inclusion complex with β-CD although their intermolecular interaction was energetically weak. For the Cys monomer, there was flat molecular surface rather than deep cavity; therefore, only one-side of fisetin was faced to the Cys monomer. The number of intermolecular close contacts between fisetin and Cys monomer was 115 which is comparable to the value of β-CD or Cys dimer (Table 1). The β-CD and Cys dimer complex ranked 136 and 151 close contacts, respectively. Moreover, their distribution was much more isotropic (Fig. 6a and c). The fisetin is able to develop stable complex with β-CD or Cys dimer because it make contacts at all over the place. However, fisetin showed quite anisotropic contact orientation, in which the fisetin use only one direction within the Cys monomer (Fig. 6b). Even though the docking score of fisetin: Cys monomer was somewhat higher than the value of fisetin: β-CD, only one-sided interaction is more readily deteriorated by third party intervention in the case of complex with Cys monomer. For the Cys dimer, both docking score and close contact with fisetin were favorable compared to the

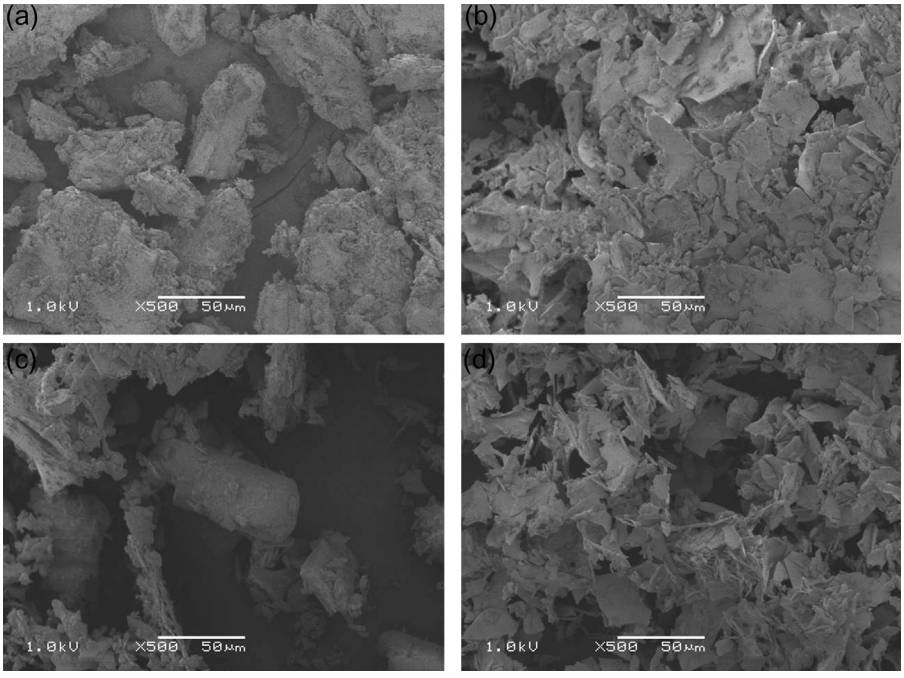

Fig. 5. Scanning electron micrographs of fisetin (a), Cys dimer (b), physical mixture (c) and fisetin–Cys dimer complex (d), 500× magnification, bar = 50 μm.

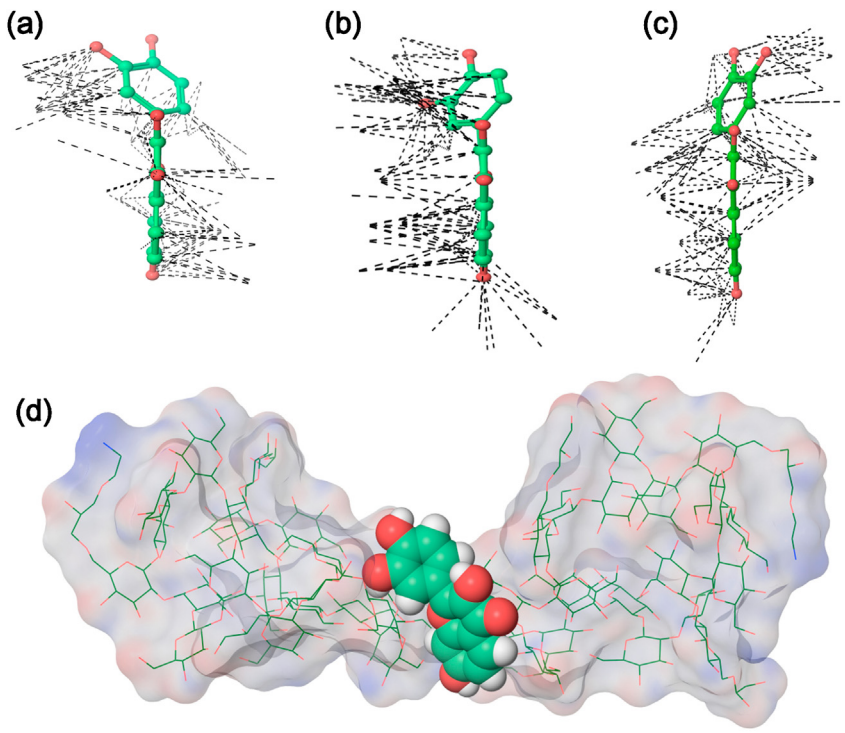

Fig. 6. Distribution pattern of intermolecular close contacts (a–c) and top-ranked docked pose for the fisetin upon Cys dimer (d). Close contacts were represented as black dotted line for the fisetin complex with β-CD (a), Cys monomer (b), and Cys dimer (c). In this case, only fisetin molecule was rendered because of visual clarity.

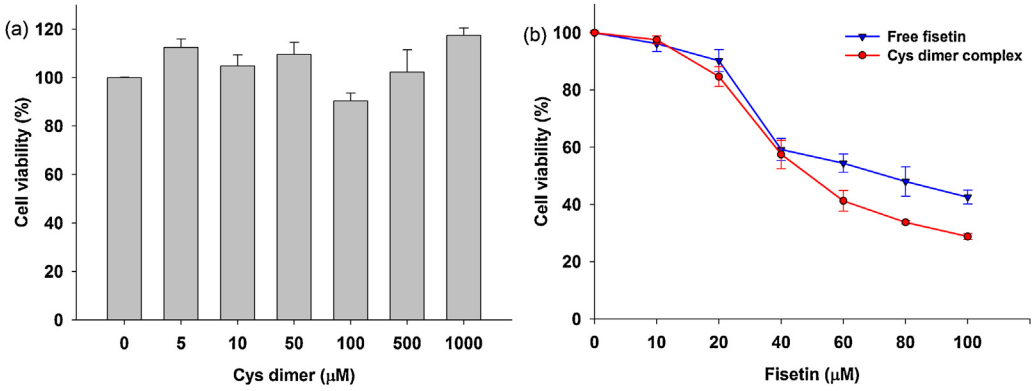

Fig. 7. Cytotoxicity of the Cys dimer (a), free fisetin, and the fisetin–Cys dimer complex (b) against HeLa cells ($n = 3$).

values of β-CD or Cys monomer. The Cys dimer is semi-linear polymer joined by cross-linking between two circular Cys monomers through 2,3-dihydroxypropoxy-coupling. The fisetin just get settled on the cross-linker interface between Cys monomer moieties, and was encircled by some glycosidic residues in the Cys dimer (Fig. 6d). Hydrogen bonds of 3′-OH and 7-OH of the fisetin with O5 and 6-OH of glucose ring of the Cys dimer were additional driving force to strengthen stability of the complex.

3.8. In vitro cytotoxicity assay

To test the cytotoxicity of the Cys dimer, HeLa cells were treated with various concentrations of Cys dimer. The results of this assay revealed that the Cys dimer was not cytotoxic to HeLa cells even at 1000 μM (Fig. 7a). This result shows that the Cys dimer can be used as a drug-complexing agent in therapeutic applications.

Next, HeLa cells were treated with free fisetin and the fisetin–Cys dimer complex at concentrations ranging from 0 to 100 μM. The cytotoxicity of fisetin and the fisetin–Cys complex toward HeLa cells differed notably (Fig. 7b). Recently, Ying et al. (2012) have reported that fisetin decreases HeLa cell viability. In this study, the fisetin–Cys dimer complex did not hinder the activity of fisetin. Furthermore, the fisetin–Cys dimer complex exhibited higher cytotoxicity against HeLa cells than free fisetin at concentrations more than 40 μM. Finally, 29% of the HeLa cells were viable after treatment with 100 μM fisetin–Cys dimer complex, whereas 43% of the HeLa cells were viable after treatment with fisetin. These results indicated that fisetin bioavailability declined because of the low aqueous solubility at high concentrations. However, the bioavailability of fisetin would increase owing to the enhanced solubility of the fisetin–Cys dimer complex.

4. Conclusions

In this study, Cys dimer was synthesized from epichlorohydrin, choline chloride, and Cys isolated from *Rhizobium* species, and complex formation with a class of flavonoid, called fisetin, was studied. The stability constant of the fisetin–Cys dimer complex

was determined to be 2188.18 M^{-1} by using the phase solubility diagram. Job's method was used to determine the stoichiometry of the Cys dimer–fisetin complex to be 1:1. The correlation between the protons of the 3 aromatic rings in fisetin and the proton of the glucose unit in the Cys dimer was assessed *via* NOESY analysis. The results indicated that in the complex, the Cys dimer wraps around the aromatic rings of fisetin. Also, the fisetin–Cys dimer complex was confirmed by using FT-IR spectroscopic, DSC and SEM analysis. Based on the molecular modeling, Cys dimer shows the better complexation ability of fisetin than that of β-CD or Cys monomer. The complexation of fisetin with Cys dimer significantly improved cytotoxicity of fisetin at higher concentrations against HeLa cell. These results confirm that Cys dimer not only enhances the water-solubility of fisetin, but also increases its bioavailability. It was suggested that the Cys dimer provides an effective approach for a more rational application of fisetin, diminishing the use of organic solvents and amount of fisetin and increasing its efficacy.

Acknowledgments

This work was supported by Priority Research Centers Program through National Research Foundation of Korea Grant funded by the Korean Government (Ministry of Education, Science and Technology (NRF 2012-0006686) and was partly supported by the National Research Foundation of Korea Grant funded by the Korean Government (NRF-2011-0026022 and NRF-2011-619-E0002). SDG.

References

Abe, M., Amemura, A., & Higashi, S. (1982). Studies on cyclic β-1,2-glucan obtained from periplasmic space of *Rhizobium trifolii* cells. *Plant and Soil*, *64*(3), 315–324.

Amemura, A., Hisamatsu, M., Mitani, H., & Harada, T. (1983). Cyclic (1→2)-β-D-glucan and the octasaccharide repeating-units of extracellular acidic polysaccharides produced by *Rhizobium*. *Carbohydrate Research*, *114*(2), 277–285.

Arai, Y., Watanabe, S., Kimira, M., Shimoi, K., Mochizuki, R., & Kinae, N. (2000). Dietary intakes of flavonols flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. *The Journal of Nutrition*, *130*(9), 2243–2250.

Chien, C.-S., Shen, K.-H., Huang, J.-S., Ko, S.-C., & Shih, Y.-W. (2010). Antimetastatic potential of fisetin involves inactivation of the PI3K/Akt and JNK signaling pathways with downregulation of MMP-2/9 expressions in prostate cancer PC-3 cells. *Molecular and Cellular Biochemistry*, *333*(1–2), 169–180.

Choi, Y.-H, Yang, C.-H., Kim, H.-W., & Jung, S. (2000). Molecular dynamics simulations of cyclohenicosakis-[(1→2)-β-D-gluco-henicosapyranosyl], a cyclic (1→2)-β-D-glucan (a 'cyclosophoraose') of DP 21. *Carbohydrate Research*, *326*(3), 227–234.

Del Valle, E. M. M. (2004). Cyclodextrins and their uses: A review. *Process Biochemistry*, *39*(9), 1033–1046.

DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350–356.

Dylan, T., Ielpi, L., Stanfield, S., Kashyap, L., Douglas, C., Yanofsky, M., et al. (1986). *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens*. *Proceedings of the National Academy of Sciences*, *83*(12), 4403–4407.

Friesner, R. A., Banks, J. L., Murphy, R. B., Halgren, T. A., Klicic, J. J., Mainz, D. T., et al. (2004). Glide: A new approach for rapid, accurate docking and scoring 1. Method and assessment of docking accuracy. *Journal of Medicinal Chemistry*, *47*(7), 1739–1749.

Geremia, R. A., Cavaignac, S., Zorreguieta, A., Toro, N., Olivares, J., & Ugalde, R. A. (1987). A *Rhizobium meliloti* mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharide but fails to form beta-(1-2) glucan. *Journal of Bacteriology*, *169*(2), 880–884.

Giordano, F., Novak, C., & Moyano, J. R. (2001). Thermal analysis of cyclodextrins and their inclusion compounds. *Thermochimica Acta*, *380*(2), 123–151.

Guzzo, M. R., Uemi, M., Donate, P. M., Nikolaou, S., Machado, A. E. H., & Okano, L. T. (2006). Study of the complexation of fisetin with cyclodextrins. *Journal of Physical Chemistry A*, *110*(36), 10545–10551.

Harada, A., Furue, M., & Nozakura, S.-i. (1980). Cooperative binding by cyclodextrin dimers. *Polymer Journal*, *12*(1), 29–33.

Higuchi, T., & Connors, K. A. (1965). Phase-solubility techniques. *Advances in Analytical Chemistry and Instrumentation*, *4*, 117–212.

Hilmi, F., Gertsch, J., Bremner, P., Valovic, S., Heinrich, M., Sticher, O., et al. (2003). Cytotoxic versus anti-inflammatory effects in HeLa Jurkat Tand human peripheral blood cells caused by guaianolide-type sesquiterpene lactones. *Bioorganic and Medicinal Chemistry*, *11*(17), 3659–3663.

Jeon, Y., Kwon, C., Cho, E., & Jung, S. (2010). Carboxymethylated cyclosophoraose as a novel chiral additive for the stereoisomeric separation of some flavonoids by capillary electrophoresis. *Carbohydrate Research*, *345*(16), 2408–2412.

Jeong, D., Piao, J., Kwon, C., & Jung, S. (2012). Synthesis of oligomeric microbial cyclosophoraoses as novel complexation agents. *Bulletin of the Korean Chemical Society*, *33*(6), 2095–2098.

Job, P. (1928). Job's method of continuous variation. *Annali di Chimica*, *10*, 113–203.

Kimira, M., Arai, Y., Shimoi, K., & Watanabe, S. (1998). Japanese intake of flavonoids and isoflavonoids from foods. *Journal of Epidemiology*, *8*(3), 168–175.

Koizumi, K., Okada, Y., Horiyama, S., Utamura, T., Higashiura, T., & Ikeda, M. (1984). Preparation of cyclosophoraose-A and its complex-forming ability. *Journal of Inclusion Phenomena*, *2*(3–4), 891–899.

Kwon, C., & Jung, S. (2011). pH-Dependent Inclusion Complexation of Highly Succinylated Cyclosophoraoses with 4'-Hydroxyflavanone. *Bulletin of the Korean Chemical Society*, *32*(8), 2791–2794.

Kwon, C., Choi, Y., Jeong, D., Kim, J., Choi, J., Chun, S., et al. (2012). Inclusion complexation of naproxen with cyclosophoraoses and succinylated cyclosophoraoses in different pH environments. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, *74*(1–4), 325–333.

Lee, S., Park, H., Seo, D., Choi, Y., & Jung, S. (2004). Synthesis and characterization of carboxymethylated cyclosophoraose, and its inclusion complexation behavior. *Carbohydrate Research*, *339*(3), 519–527.

Li, J., Xiao, H., Li, J., & Zhong, Y. (2004). Drug carrier systems based on water-soluble cationic β-cyclodextrin polymers. *International Journal of Pharmaceutics*, *278*(2), 329–342.

Lipinski, C. A. (2000). Drug-like properties and the causes of poor solubility and poor permeability. *Journal of Pharmacological and Toxicological Methods*, *44*(1), 235–249.

Misiuk, W., & Zalewska, M. (2009). Investigation of inclusion complex of trazodone hydrochloride with hydroxypropyl-β-cyclodextrin. *Carbohydrate Polymers*, *77*(3), 482–488.

Okada, Y., Horiyama, S., & Koizumi, K. (1986). Studies on inclusion complexes of non-steroidal anti-inflammatory agents with cyclosophoraose-A. *Yakugaku Zasshi*, *106*(3), 240–247.

Park, H., Lee, S., Kang, S., Jung, Y., & Jung, S. (2004). Enantioseparation using sulfated cyclosophoraoses as a novel chiral additive in capillary electrophoresis. *Electrophoresis*, *25*(16), 2671–2674.

Ragelle, H., Crauste-Manciet, S., Seguin, J., Brossard, D., Scherman, D., Arnaud, P., et al. (2012). Nanoemulsion formulation of fisetin improves bioavailability and antitumour activity in mice. *International Journal of Pharmaceutics*, *427*(2), 452–459.

Samal, S. K., Dash, M., Van Vlierberghe, S., Kaplan, D. L., Chiellini, E., van Blitterswijk, C., et al. (2012). Cationic polymers and their therapeutic potential. *Chemical Society Reviews*, *41*(21), 7147–7194.

Schneider, H.-J., Hacket, F., Rüdiger, V., & Ikeda, H. (1998). NMR studies of cyclodextrins and cyclodextrin complexes. *Chemical Reviews*, *98*(5), 1755–1786.

Spaink, H. (1992). Rhizobial lipo-oligosaccharides: Answers and questions. *Plant Molecular Biology*, *20*(5), 977–986.

Szente, L. (1996). Cyclodextrins. In J. Szejtli, & T. Osa (Eds.), *Comprehensive supramolecular chemistry* (pp. 253–278). Oxford, UK: Pergamon.

Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiological Reviews*, *56*(3), 395–411.

Wen, J., Liu, B., Yuan, E., Ma, Y., & Zhu, Y. (2010). Preparation and physicochemical properties of the complex of naringenin with hydroxypropyl-β-cyclodextrin. *Molecules*, *15*(6), 4401–4407.

Xiao, H., & Cezar, N. (2005). Cationic-modified cyclodextrin nanosphere/anionic polymer as flocculation/sorption systems. *Journal of Colloid and Interface Science*, *283*(2), 406–413.

Yang, L.-J., Chen, W., Ma, S.-X., Gao, Y.-T., Huang, R., Yan, S.-J., et al. (2011). Host–guest system of taxifolin and native cyclodextrin or its derivative: Preparation, characterization, inclusion mode, and solubilization. *Carbohydrate Polymers*, *85*(3), 629–637.

Ying, T.-H., Yang, S.-F., Tsai, S.-J., Hsieh, S.-C., Huang, Y.-C., Bau, D.-T., et al. (2012). Fisetin induces apoptosis in human cervical cancer HeLa cells through ERK1/2-mediated activation of caspase-8-/caspase-3-dependent pathway. *Archives of Toxicology*, *86*(2), 263–273.