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

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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.

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

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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,