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Solubility enhancement of α-naphthoflavone by synthesized hydroxypropyl cyclic-(1→2)-β-D-glucans (cyclosophoroases)

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ABSTRACT

Rhizobium leguminosarum produces unbranched cyclic β-1,2-glucans, cyclosophoraoses (Cys). In the present study, Cys were modified with hydroxypropyl groups via a one step chemical derivatization and the complexation ability and solubility enhancement of hydroxypropyl cyclosophoraoses (HP Cys) with α-naphthoflavone (α-NF) were investigated. In the presence of HP Cys, the aqueous solubility of α-NF greatly increased up to 257-fold. Complex formation of HP Cys and α-NF was confirmed by nuclear magnetic resonance (NMR), Fourier-transform infrared (FT-IR) spectroscopy, and differential scanning calorimetry (DSC). Furthermore, the morphological structure of α-NF with HP Cys was examined using scanning electron microscopy (SEM). A hypothetical model was proposed based on molecular dynamics (MD) simulations and a docking study of α-NF with HP Cys. Our results suggest that HP Cys form complexes with α-NF and can be utilized as a promising solubilizer. This is the first study to identify carbohydrates that can enhance the solubility of α-NF.

1. Introduction

Cyclosophoraoses (Cys) are a class of unbranched cyclic oligosaccharides isolated from *Rhizobium leguminosarum biovar viciae* VF-39. They are synthesized in the periplasm and transported to the extracellular space, playing an important role in osmoregulation and root nodule formation during nitrogen fixation (Zhang, Hollingsworth, & Priefer, 1992). Cys are cyclic glucans composed of glucose residues linked by β-1,2-glycosidic bonds, varying in size from 17 to 23 in their degree of polymerization (DP). Because Cys can form small cavities (André, Mazeau, Taravel, & Tvaroska, 1995), they have been investigated for complexation techniques. Cys have the ability to form complexes with a variety of hydrophobic guest molecules, such as indomethacin, luteolin, and paclitaxel (Lee, Seo, Park, Choi, & Jung, 2003; Lee et al., 2001a; Lee, Seo, Kim, & Jung, 2001b). They can also be modified with various functional groups, such as carboxy-methyl, butyryl, and methyl groups. Recent reports have shown that these functionalized Cys have increased complexation ability with guest molecules (Kwon, Cho, Lee, & Jung, 2011; Lee, Park, Seo, Choi, & Jung, 2004).

Another cyclic oligosaccharide, cyclodextrin (CD), has been reported to have inclusion complexation behavior due to its three-dimensional toroidal shape by seven α-1,4-linked glucoses (Loftsson & Masson, 2001). Notably, hydroxypropyl cyclodextrin (HP CD) has shown increased inclusion complexing ability and water solubility as well as lower toxicity compared to that of native CD. In addition, the attached hydroxypropyl groups could provide additional space for larger compounds (Morillo et al., 2012; Shulman et al., 2011). The complexation ability of HP CD improves the bioavailability of the hardly soluble drugs. Therefore, we synthesized HP Cys, which are composed of β-1,2 linkages and a higher number of glucose units, to make a novel host molecule for bulky guest compounds. The final structures of HP Cys were analyzed by nuclear magnetic resonance (NMR) spectroscopy and matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry.

Flavonoids are one of the major active nutraceutical ingredients in plants and over 8000 different flavonoids have been identified. They exert various physiological effects, including antioxidant,

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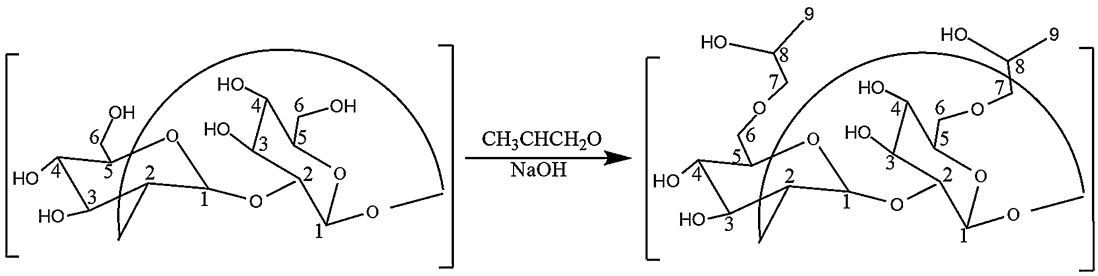

Scheme 1. Synthesis of HP Cys.

anticarcinogenic, and antiestrogenic effects (Chu, Chang, & Hsu, 2000; Collins-Burow et al., 2012; Ren, Qiao, Wang, Zhu, & Zhang, 2003). To expand their functionality, flavonoid derivatives have been synthesized (Chen et al., 2010). The synthetic flavonoid, α-naphthoflavone (α-NF) is a well-known potent inhibitor of aromatase (He et al., 2013), a cytochrome P450 enzyme involved in the biosynthesis of estrogen, which is the main stimulant of breast cancer cell growth. α-NF needs to be studied to understand its role in the treatment of breast cancer. Recent reports also demonstrate that α-NF has anti-aging effects on human skin fibroblasts and inhibits pre-adipocyte differentiation via modulating p38MAPK signaling (He et al., 2013; Liao et al., 2012). However, the extremely low aqueous solubility of α-NF could limit many potential clinical uses. There are no reports on molecular complexation using CD or its derivatives that increase the solubility of α-NF due to its large polyaromatic ring structure. In order to enhance the aqueous solubility of α-NF as a guest, its corresponding host needs to have more molecular space for efficient complexation. Here, newly synthesized HP Cys have shown potential as novel complexing agents with α-NF to increase its aqueous solubility.

2. Experimental

2.1. Materials

α-Naphthoflavone, propylene oxide [≥99.5% (GC)], and β-cyclodextrin (≥97%) were purchased from Sigma–Aldrich Chemicals Co. (St. Louis, MO, USA). D_2O (99.9 atom % D) and dimethyl sulfoxide-d_6 (99.9 atom % D) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). All other chemicals were of reagent grade and used without further purification.

2.2. Preparation of Cys

R. leguminosarum biovar viciae VF-39 was grown in 500 mL of GMS medium with 5 g mannitol and 150 mM of sodium chloride at 25 °C for 14 days. Cells and cultured supernatant were separated by centrifugation at 8000 × *g* for 15 min. The supernatant was concentrated 5-fold by rotary evaporation. The concentrated samples were precipitated by adding three volumes of ethanol and clarified by centrifugation at 8000 × *g* for 15 min. The supernatant was concentrated 5-fold again by rotary evaporation. Cys was then precipitated by adding another seven volumes of ethanol and clarified by centrifugation (Breedveld, Zevenhuizen, & Zehnder, 1991). The resulting precipitate was dissolved in distilled water and chromatographed on Bio-Gel P6. After concentration and desalting with Bio-Gel P-2 columns, fractions were assayed using the phenol–sulfuric acid method. Purified Cys were confirmed by MALDI-TOF mass spectrometry (Voyager-DETM STR Bio-Spectrometry, Applied Biosystems, Framingham, MA, USA) in the positive ion mode using 2,5-dihydroxybenzoic acid (DHB) as the matrix. NMR spectroscopy (Bruker 500 MHz spectrometer, AMX, Germany) was also used to confirm Cys in D_2O solvent.

2.3. Synthesis of HP Cys

HP Cys were synthesized in a one-step reaction (Scheme 1). Sodium hydroxide (295.7 mg) was dissolved in 822 μL of distilled water. Cys (200 mg) were added to the sodium hydroxide solution with stirring until completely dissolved. Propylene oxide (520 μL) was evenly added in 6 min at freezing point with stirring. After the mixture was reacted for 24 h at room temperature, 5N HCl was added to stop the reaction (pH = 7) (Pitha, Szabo, & Fales, 1987). A Bio-Gel P-4 column was used to desalt the mixture and fractions assayed using the phenol–sulfuric acid method were distilled. The resulting thick slurry was freeze-dried to obtain purified HP Cys, which were confirmed by NMR spectroscopy and MALDI-TOF mass spectrometry.

2.4. Phase solubility study

Phase solubility studies were performed using the method described by Higuchi and Connors (Higuchi & Connors, 1965). An excess amount of α-NF (2 mM) was added to various concentrations of HP Cys and β-CD (0.0 to 8.0 mM). The mixtures were magnetically stirred for 24 h at 25 °C in capped vials to prevent degradation of the molecules by light. After reaching equilibrium, samples were filtered through a 0.2 μm PVDF syringe filter and analyzed by ultraviolet–visible (UV–vis) spectrometry (UV 2450, Shimadzu Corporation) from 200 to 350 nm. The stability constant (K_c) of α-NF/HP Cys complexes was calculated using the following equation based on the phase solubility diagram:

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

2.5. Continuous variation method

The stoichiometry of α-NF and HP Cys were determined using the continuous variation method (Hill & MacCarthy, 1986). The total concentrations of α-NF and HP Cys were kept constant at 1 mM. The absorbance difference between samples with and without HP Cys was plotted as a function of the α-NF mole fraction.

2.6. FT-IR spectroscopic analysis

FTIR spectroscopy (AMX, Germany) was performed in the range of 4000–500 cm^{-1} in a KBr matrix. Spectra were obtained for α-NF, HP Cys, α-NF/HP Cys physical mixtures, and the α-NF/HP Cys complexes.

2.7. Differential scanning calorimetry (DSC)

Thermal analyses of the samples were performed using differential scanning calorimetry DSC Q200 V24.4 (TA Instruments, USA). Samples were heated in a sealed aluminum pan over a temperature range of 50–300 °C at a rate of 10 °C min^{-1} and an empty sealed pan

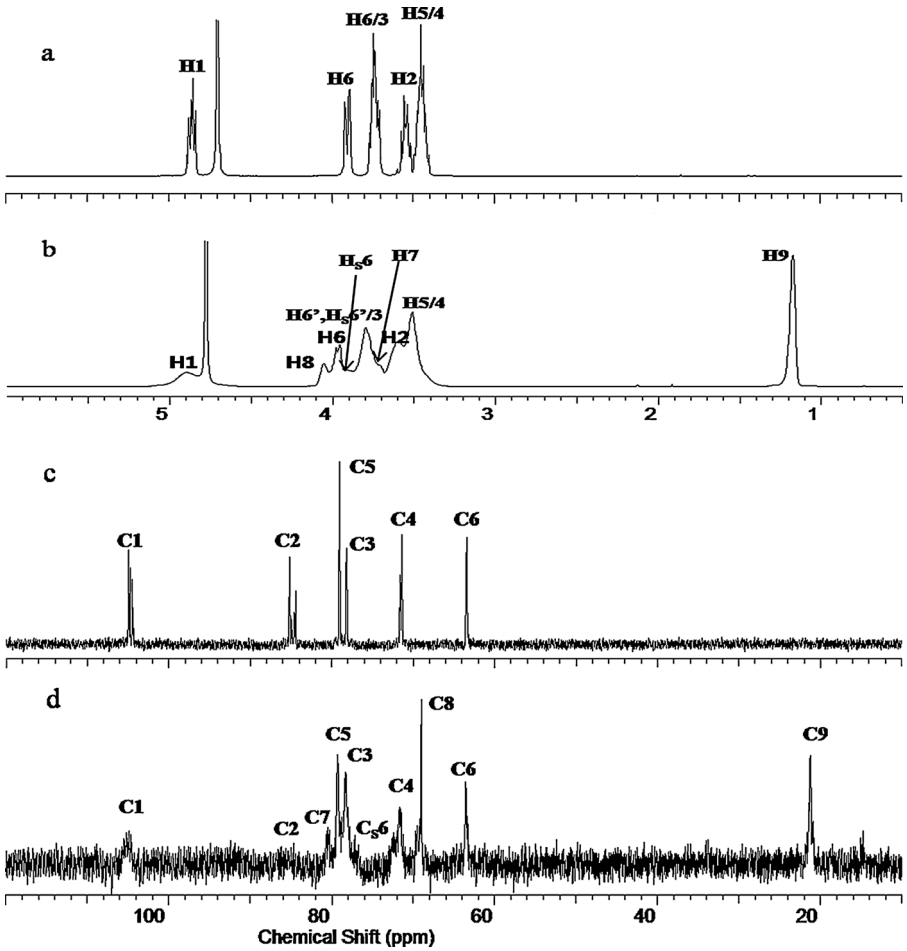

Fig. 1. ^{1}H NMR spectra of (a) Cys and (b) HP Cys. ^{13}C NMR spectra of (c) Cys and (d) HP Cys.

was used as a reference. An indium standard was used to calibrate the temperature scale.

2.8. Nuclear magnetic resonance (NMR) spectroscopy

For the NMR spectroscopic analysis, we used a Bruker Avance 500 spectrometer to perform nuclear overhauser effect spectroscopy (NOESY). The NOESY spectrum was recorded with 256/2048 complex data points using a pulse train to achieve a spin-lock field with a mixing time of 600 ms for the complex. Samples were dissolved in 70% dimethyl sulfoxide-d_6 (99.9 atom % D) and 30% deuterated water (99.9 atom % D)

2.9. Scanning electron microphotographs (SEM)

The surface morphology of materials was examined using a JSM-6380 scanning electron microscope. Powders were fixed on a brass stub using double-sided adhesive carbon tape and then made electrically conductive by coating a thin layer of gold on the surface. Pictures were taken at an excitation voltage of 20 kV.

2.10. Computational method

A structural model for HP Cys was constructed using Discovery Studio 2.5 product in Accelrys software. At first, the original Cys

Table 1
Measured and calculated mass units of the m/z peaks of HP Cys.

TNS	DP						
	17	18	19	20	21	22	23
11		3579 (3554)	3742 (3716)	3927 (3878)			
12		3637 (3612)	3799 (3774)	3986 (3936)			
13		3696 (3670)	3858 (3832)	4043 (3994)			
14			3916 (3890)	4102 (4052)	4264 (4214)		
15			3975 (3948)	4160 (4110)	4322 (4272)		
16			4032 (4006)	4218 (4168)	4380 (4330)		
17			4090 (4064)	4276 (4226)	4438 (4388)	4601 (4550)	4763 (4712)
18			4148 (4122)		4496 (4446)	4658 (4608)	4821 (4770)
19			4206 (4180)		4554 (4504)	4716 (4666)	4879 (4828)
20							4936 (4886)
21							4994 (4944)
22							5053 (5002)

TNS: total number of substitution; DP: degree of polymerization. The numbers in parentheses indicate theoretically calculated values.

(*DP* = 19) structure was chemically modified with a hydroxypropyl group on each 6′-OH group of glucose residues. The initial structure of HP Cys was calculated using a general conformation search and the Boltzmann jump protocol at 300 K. The protocol was performed using a consistent force field (CFF) with an implicit generalized born (GB) environment. The structure of HP Cys was energy-minimized and subjected to a macrocyclic conformational sampling tool that incorporates a standard dynamics cascade with CFF. There were 5 phases in the tool. First, two phases were carried out using energy minimization processes consisting of the steepest descent and conjugate gradient algorithms. Third, target temperatures in heating phase increased by 1000 K from 300 K within 6 ps. Next, the equilibration phase was for 10 ps at each temperature toward 300 K. Finally 1000 ps of production molecular modeling (MD) runs in NVT-ensemble were performed at 300 K. In these phases of MD simulations, 1 fs of time step was applied (Choi, Yang, Kim, & Jung, 2000).

A flexible-ligand docking job of α-NF on a host molecule was performed by CDOCKER of the Discovery Studio 2.5 product in Accelrys software. The starting structure of α-NF was prepared from a molecular sketch and general conformation searching of the interface.

As a grid-based MD docking algorithm, CDOCKER (CHARMm-based DOCKER) uses a simulated annealing method by heating and cooling jobs between 2000 steps of runs at 700 K and 5000 steps of runs at 300 K. Each docked pose was fully energy-minimized before further analysis.

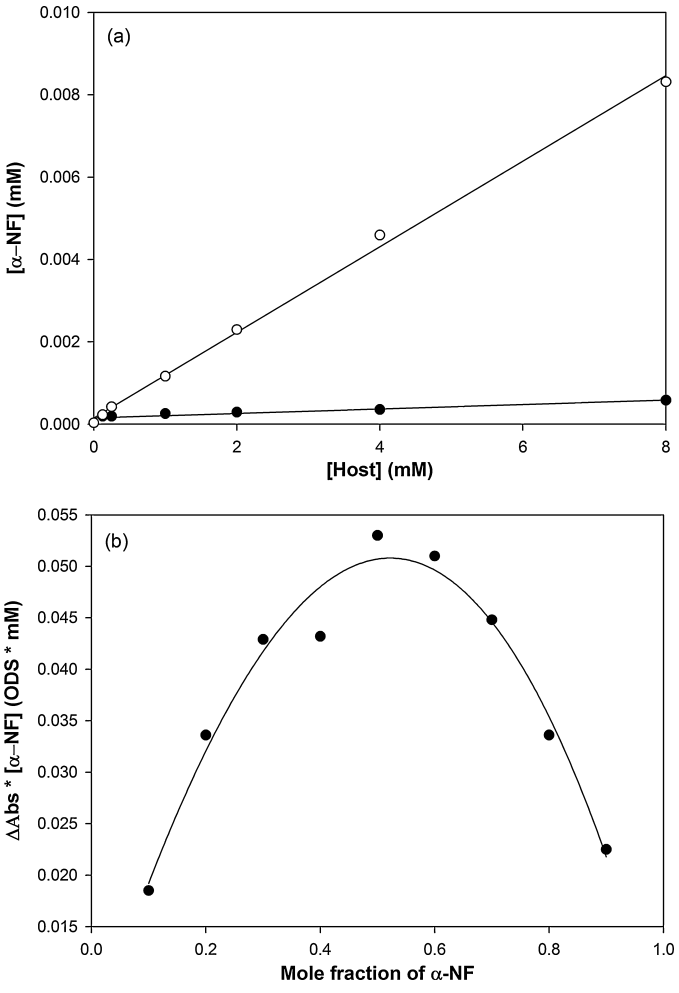

Fig. 2. (a) Phase solubility diagram of α-NF with β-CD (●) and HP-Cys (○) complexes in water at 25 °C. The concentrations of HP Cys were 0 mM, 0.125 mM, 0.25 mM, 1 mM, 2 mM, 4 mM, and 8 mM. (b) Job's plot of the α-NF/HP Cys complex.

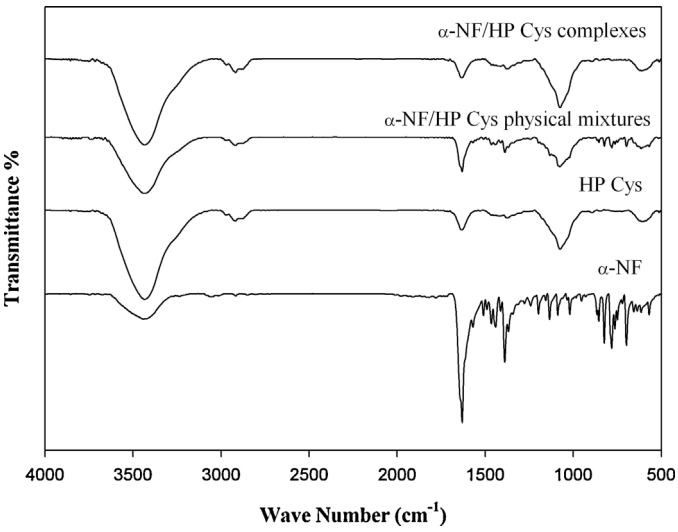

Fig. 3. FT-IR spectra of α-NF/HP Cys complexes, physical mixtures, HP Cys, and α-NF.

3. Results and discussion

3.1. Structural analyses of HP Cys

In the ^{1}H NMR spectra, protons of native Cys and HP Cys were assigned (Fig. 1a and b). Compared to native Cys, new peaks were detected in HP Cys. The peaks between 1.10 ppm and 1.30 ppm correspond to CH_3—CHOH—CH_2—O— of the hydroxypropyl residues. Peaks from 3.60 to 3.65 ppm correspond to CH_3—CHOH—CH_2—O— of the hydroxypropyl residues and peaks ranging from 3.95 to 4.00 ppm correspond to CH_3—CHOH—CH_2—O— of the hydroxypropyl residues (Pitha, Milecki, Fales, Pannell, & Uekama, 1986).

^{13}C NMR spectra indicated that the hydroxypropyl substituents are attached to C-6 carbons of the glucose unit, and substituted C-6′ carbons are present (73 ppm). Also, the peaks at 20–22 ppm correspond to CH_3—CHOH—CH_2, signals at 68–70 ppm belong to the CH_3—CHOH—CH_2, and peaks at 80–82 ppm correspond to CH_3—CHOH—CH_2 of the hydroxypropyl residue (Fig. 1c and d). NMR spectroscopic analyses indicate that hydroxypropyl residues are successfully substituted at the C-6th positions of native Cys.

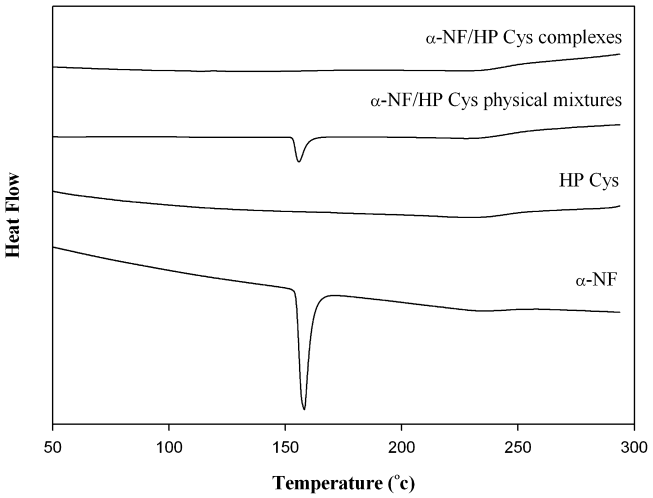

Fig. 4. DSC curves of α-NF/HP Cys complexes, physical mixtures, HP Cys, and α-NF.

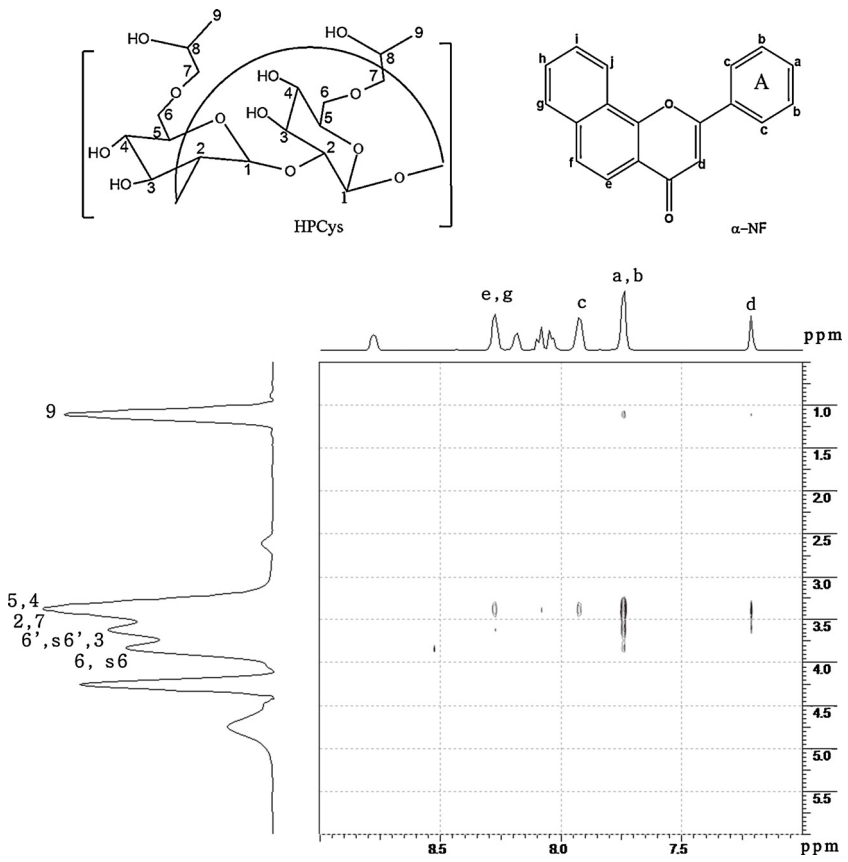

Fig. 5. NOESY spectrum of a solution containing 70% dimethyl sulfoxide-d_6 (99.9 atom % D), 30% deuterated water (99.9 atom % D), and equimolar amounts of α-NF (10 mM) and HP Cys (10 mM).

The measured mass units of HP Cys appeared as $[M + Na^+ + 2H^+]$, $[M + Na^+ + 3H^+]$, $[M + Na^+ + 4H^+]$, $[M + 2Na^+ + 4H^+]$, and $[M + 2Na^+ + 5H^+]$ (Table 1). Depending on the DP range of Cys, HP Cys exhibited a different degree of substitution (DS) value. The highest peak in the mass data appeared at m/z 4206, indicating [HP Cys (DP 19 and DS 19) + $Na^+ + 4H^+$]. The average DS of HP Cys was determined to be around 22, and the average molecular weight was calculated to be 4393. The newly synthesized HP Cys with additional three-dimensional space by the attached hydroxypropyl groups on the Cys backbone should have increased complexing

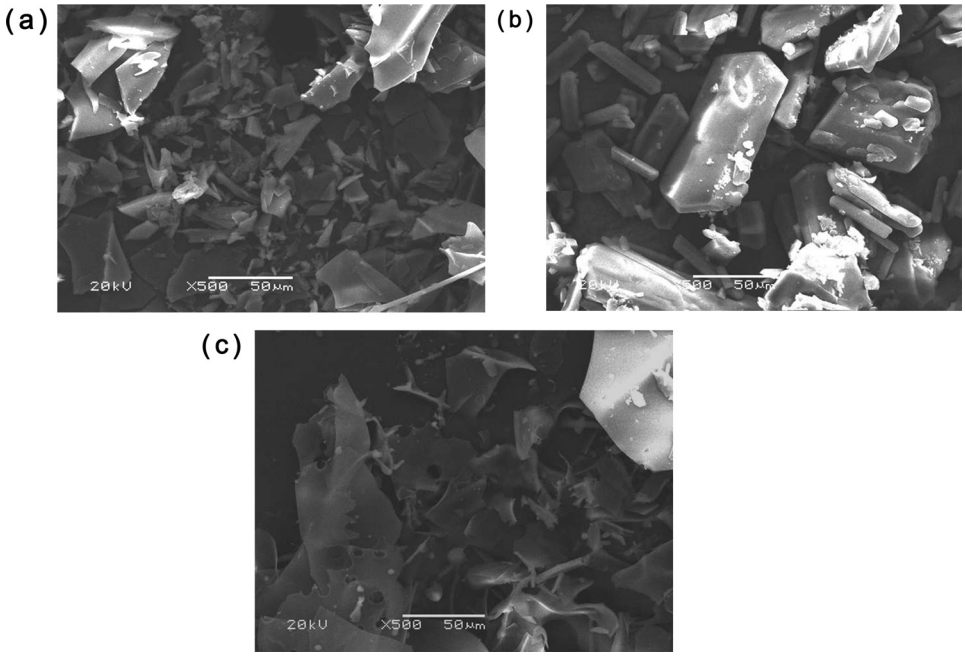

Fig. 6. Scanning electron micrographs of (a) α-NF, (b) HP Cys, and (c) α-NF/HP Cys complexes (1:1 molar ratio) using 500× magnification. Bar = 50 μm.

ability with bulky hydrophobic compounds, such as α-NF (Pitha, Milecki, Fales, Pannell, & Uekama, 1986).

3.2. Phase solubility studies

The phase solubility diagram of α-NF/HP Cys shown in Fig. 2a indicates that the complex formed A_L type curves (Martin Del Valle, 2004). The solubility of α-NF improved up to 257-fold after the addition of 8 mM HP Cys and 18-fold in the presence of 8 mM β-cyclodextrin. From the phase solubility diagram, the stability constant (K_c) of α-NF/HP Cys complex is calculated as 33,963 M^{-1}, whereas that of α-NF/β-CD complex is 2472 M^{-1}. These results indicate that HP Cys are effective solubility enhancers for α-NF. sults indicate that HP Cys are effective solubility enhancers for α-NF.

3.3. Continuous variation method

Stoichiometry was confirmed using the continuous variation method (Job's plot). Continuous variations are commonly used for determining the composition of complexes in solution (Loftsson, Magnúsdóttir, Másson, & Sigurjónsdóttir, 2002). The absorbance was maximal at a mole fraction of 0.5 (Fig. 2b), which indicates a stoichiometry of 1:1 (HP Cys: α-NF). This result is in agreement with an A_L type of phase solubility diagram.

3.4. FT-IR spectroscopic analysis

The variation of the shape, shift, and intensity of FT-IR absorption peaks of the guest or host can provide enough information about the formation of complexes (Patil, Ingole, Singh, & Dalal, 2012). The physical properties of α-NF, HP Cys, the α-NF/HP Cys physical mixtures, and α-NF/HP Cys complexes were investigated by FT-IR (Fig. 3). The FT-IR spectrum of α-NF showed characteristic absorption bands for carbonyl stretching vibration at 1631 cm^{-1}, aromatic C=C stretching at 1359–1541 cm^{-1}, and an aromatic C—H out-of-plane bend at 700–865 cm^{-1}. The intensities of the characteristic absorption bands were slightly decreased in the physical mixture due to the peak overlapping of HP Cys and α-NF. Several reports on decreasing intensities of guest peaks in the physical mixtures have already been published (Hu, Zhang, Song, Gu, & Hu, 2012; Wang, Ouyang, Liu, Yuan, & Liu, 2013; Zhang et al., 2013). However, these characteristic absorption peaks completely disappeared in α-NF/HP Cys complexes, confirming α-NF/HP Cys complexation.

3.5. Differential scanning calorimetry (Table 2)

DSC was performed to characterize the solid state interactions of inclusion complexes (Pinzaru et al., 2011). Thermograms for α-NF, HP Cys, α-NF/HP Cys physical mixtures, and α-NF/HP Cys complexes were obtained by DSC. The melting endothermic peak of α-NF at 156 °C disappeared in the α-NF/HP Cys complex, indicating that α-NF forms complexes with HP Cys (Fig. 4).

Table 2
List of close contact distances between α-NF and HP Cys.

Close contact list		
α-NF	HP Cys	Distance [Å]
a	9H′ (i – 2)	2.48
b	6H′ (i – 1)	4.364
b	9H′ (i + 6)	2.641
e	4H′ (i + 3)	4.336

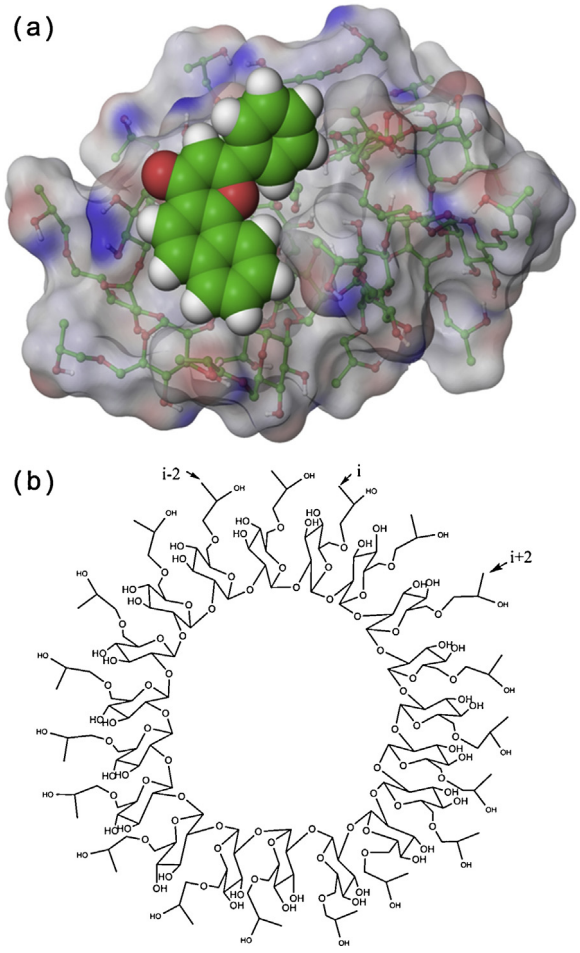

Fig. 7. A representative docking conformation of α-NF complexed with HP-Cys19. (a) A snapshot of a docking pose. α-NF is represented using a space filling model and the HP Cys structure is shown as a ball-and-stick as a solvent accessible surface area in shadow. Green is carbon, red is oxygen, and polar hydrogens are white. The other atoms are omitted. (b) A scaffold of HP Cys with labeled rings by indexing i. Arrows of i indicate the methyl groups for hydrophobic interactions mainly. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.6. Nuclear magnetic resonance (NMR) spectroscopy

To confirm the interactions between α-NF and HP Cys, nuclear overhauser enhancement spectroscopic (NOESY) analysis was performed. Two-dimensional NMR spectroscopy has recently become an indispensable method for examining interactions between guest and host molecules. If an NOE cross peak is detected between protons of interest, then one can conclude that the two protons are closely located within a distance of 5 Å (Liu et al., 2001). The a–e, and g protons of α-NF showed correlation NOE cross peaks with HP Cys protons (Fig. 5). The strongest correlation occurred between a, b protons of α-NF and 5, 4 protons of HP Cys. These results demonstrate that the protons of α-NF and HP Cys closely interact, further indicating α-NF/HP Cys complexation.

3.7. Scanning electron microphotographs

SEM helps to assess the existence of a single component in the preparations obtained based on the morphological changes that occur in the presence or absence of complexation (Baboota, Dhaliwal, & Kohli, 2005). α-NF exhibited regular shaped crystals, whereas HP Cys were present as amorphous particles (Fig. 6a–b). After complexation, the morphology appeared to be quite different

from the sizes and shapes of both free α-NF and HP Cys (Fig. 6c), providing additional evidence of complex formation.

3.8. Computational analysis

Fig. 7 is a representative snapshot view of the docked poses of α-NF molecules upon HP Cys. The docking results provide CDOCKER energy as well as interaction energy, which includes internal ligand strain energy and the non-bonded interaction energy between receptor and ligand. The hydroxypropyl groups of HP Cys formed enough nest-like structures to accommodate α-NF (Fig. 7a). In particular, the hydrophobic triad formed by three hydroxypropyl groups on top of i, i+2, and i−2 sugar rings seems to be mainly responsible for holding of α-NF (Fig. 7b). The flexible poses between α-NFs upon HP Cys from the docking result indicate variable interactions. The Table 2 lists the contact distances between α-NF and HPCys. The a, b protons on the A-ring of α-NF closely interact with the 6, 9 protons of the hydrocarbon in HP Cys. As predicted from the docking poses, the pairs within close contact were observable near hydrophobic triads formed by a specific arrangement of hydroxypropyl groups in the i, i+2, and i−2 residues of HP Cys. As a result of the highly hydrophobic methyl moiety in the hydroxypropyl groups, *van der Waals* attractions are a major driving force for the binding of α-NFs to HP Cys (Upadhyay & Kumar, 2009).

4. Conclusions

In this study, a class of unbranched cyclic oligosaccharides (Cys) was isolated from *R. leguminosarum* and successfully modified with hydroxypropyl functional groups. Complexation characteristics of HP Cys with α-NF were analyzed by NMR, FTIR, and DSC. In addition, the nature of the complexes were examined by SEM. HP Cys efficiently enhanced the solubility of α-NF compared to β-CD. The stability constants determined from phase solubility studies also suggest that HP Cys/α-NF complexes are more stable than β-CD/α-NF complexes. In addition, a 1:1 stoichiometry was shown between HP Cys and α-NF using the continuous variation method. The complexation of HP Cys and α-NF occurred via non-covalent interactions, such as *van der Waals* interactions, and hydrogen bonding. Therefore, we conclude that HP Cys are useful solubility enhancing agents that form complexes with α-NF. Molecular modeling indicated that HP Cys form enough nest-like structures to accommodate α-NF and the hydrophobic three hydroxypropyl groups on top of i, i+2, and i−2 sugar rings appear to be mainly responsible for holding α-NF. Moreover, we suggest that HP Cys function as novel host molecules with the ability to efficiently interact with various polyaromatic compounds as well as α-NF.

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