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pH-Dependent Inclusion Complexation of Highly Succinylated Cyclosophoraoses with 4'-Hydroxyflavanone

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Cyclosophoraoses (Cys) are unique molecules that are produced by all members of the family Rhizobiaceae. They were originally found not only in the periplasmic space but also in the extracellular media of *Agrobacterium* and *Sinorhizobium* species as fast-growing soil bacteria.[1] In *Agrobacterium* and *Sinorhizobium* species, they are a family of unbranched cyclic β-1,2-glucans and the predominant ring size distribution is between 17 and 25 glucose residues.[2,3] They have also been reported to be involved in nitrogen fixation,[4] attachment of bacterial cells to plant hosts[5] and osmotic regulation.[6] They have attracted considerable attention because of their potential ability to form inclusion complexes with other hydrophobic molecules as well as on their various biological functions.[7] Recent reports have shown that neutral cyclosophoraoses or anionic cyclosophoraoses have good potential as host molecules in various inclusion complexation technologies, such as novel encapsulating agents and solubility enhancers for the poorly soluble molecules.[8,9] To further applications of cyclosophoraoses, various modified cyclosophoraoses have also been used as a good host for inclusion complexation.[9] Some reports on succinyl cyclooligosacchride have also shown that the succinate moieties were significantly involved in inclusion complexation process.[23]

Flavonoids are an important class of phenolic plant constituents with beneficial health-related properties and commonly distribute in fruits and vegetables. In nature, they are available as flavone, flavonol, flavanone, isoflavone, chalcone and their derivatives.[10] Much attentions has thus been focused on diverse biological activities of natural and synthetic flavanones. Among them, 4'-hydroxyflavanone, a synthetic analogue of flavanones, is involved in various biological activities and physiological effects such as endothelium-independent full vasorelaxing efficacy on rat aortic rings,[11] aromatase inhibitor properties,[12] decreasing collagen concentration in human dermal fibroblasts[13] and moderate binding ability to the rat uterine estrogen receptor.[14] Although their diverse physiological effects, the potential clinical utility is limited as their low aqueous solublility.[15]

In the present study, succinylated cyclsophoraoses (S-Cys) were synthesized for further applications of cyclosophoraoses (Cys) by a facile chemical reaction with succinic anhydride and 4-(dimethylamino)pyridine (DMAP) in pyridine. Since the weak acidic succinyl groups attached to S-Cys show the different charge distributions depending on the surrounding pH conditions, the inclusion complexation between S-Cys as host molecules and 4'-hydroxyflavanone as a hydrophobic compound were investigated at two different pH values.

The succinylated cyclsophoraoses (S-Cys) were synthesized through a facile one-step reaction.[16] The reaction was monitored on TLC and the products were purified by the ion-exchange and size-exclusion gel chromatographic methods. Purified native Cys and synthesized S-Cys were separated with an R_f value of 0.2 and 0.35 on TLC, respectively. In addition, the ring sizes of the Cys, which ranged from 17 to 25, were confirmed through matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. We then conducted structural analyses of the Cys as described elsewhere.[8] ^{1}H NMR analysis also confirmed that the S-Cys contains a high level of succinate with the presence of two prominent signals between 2.6 and 2.8 ppm (Fig. 2(c)). Compared with native Cys, some newly detected peaks appeared at 4.5 ppm in S-Cys. This indicated that S-Cys were modified at 6- and 4-positions of glucose in the Cys by succinyl groups. The ^{1}H NMR analysis revealed S-Cys was substituted even at 3-position of glucose in the Cys by the succinyl reaction. The mass spectrum of S-Cys also represents highly succinylated cyclosophoraoses in Figure 2(b). As the average molecular weight of Cys is 3119, molecular ion peak of S-Cys appeared at 8925.91 Da. [M+Na$^+$] (*m/z* 8925.91), [2M+Na$^+$] (*m/z* 16729.20), [3M+Na$^+$] (*m/z* 25975.02) and [4M+Na$^+$] (*m/z* 35027.50) ions were also appeared in the Figure 2(b). Detailed analysis of the S-Cys revealed they were mainly modified with fifty-four succinyl

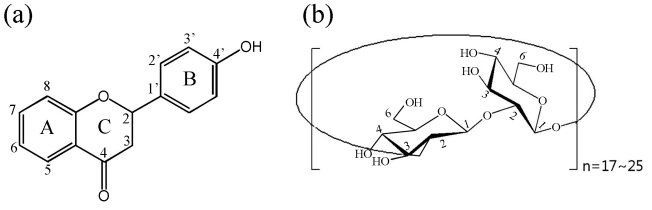

Figure 1. Chemical structures of 4'-hydroxyflavanone (a), neutral cyclosophoraose (Cys) (b) involved in this work.

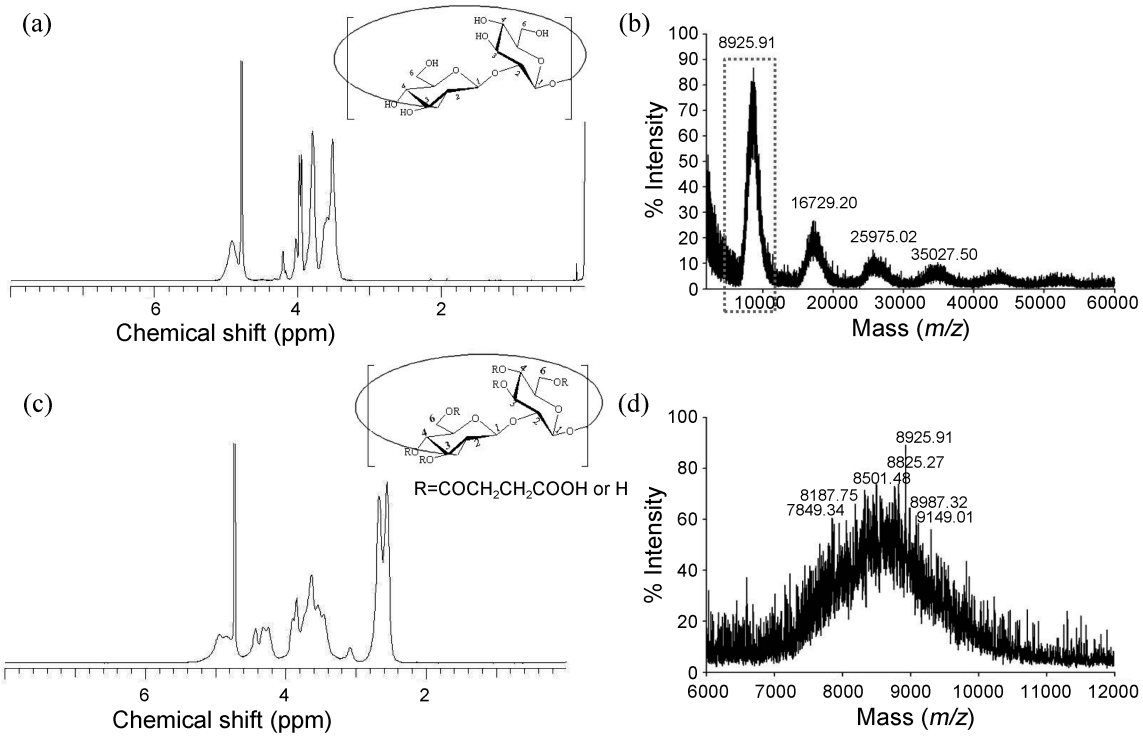

Figure 2. ^{1}H NMR spectra of netural cyclosophoraoses of *Sinorhizobium trifolii* TA-1 (a) and succinylated cyclosophoraoses by chemical modification (c). Positive ion MALDI TOF mass spectrum of succinylated cyclosophoraoses by chemical modification (b) and enlarged spectrum of *m/z* 6,000-12,000 (d).

groups (Fig. 2(d)). For example, four sodiated molecular ions, $[M+Na]^+$, appeared at *m/z* 8501.48, 8825.27, 8987.32 and 9149.01, indicating [fifty-four succinylated Cys (DP 19, 21, 22 and 23)+Na^+]. The number average molecular weight of S-Cys was determined as 8718.6, based on the MALDI-TOF mass spectrometric analysis.[17]

The inclusion complexes between S-Cys as host molecules and 4'-hydroxyflavanone as a hydrophobic compound were investigated by ^{1}H NMR spectroscopy. The proton NMR spectra of 4'-hydroxyflavanone before and after the complexation with the S-Cys showed the clear changes in chemical shifts of 4'-hydroxyflavanone and S-Cys due to the complexation. To investigate the effect of pH on the complexation capcity of S-Cys, we determined the binding constants (K_b) of 4'-hydroxyflavanone as a guest with the S-Cys. All the binding parameters were calculated on the basis of the H5, H2' and H8 protons of 4'-hydroxyflavanone. The binding constants (K_b at pH 3.4 and 9.2) were determined from the ^{1}H NMR titration curves at pH 3.4 and 9.2, and the curves were analyzed by a Benesi-Hildebrand method.[18] Figure 3 showed Benesi-Hildebrand plots for the complexes of 4'-hydroxyflavanone with the S-Cys. The equations of the lines for the complexes gave fine correlation coefficients, r^2 values, ranging from 0.975 to 0.997. Table 1 listed the averaged K_b values (K_{av}) from the equations of the complexes's plots. In previous report,[9b] carboxymethylated cyclosophoraoses (CM-Cys) which had a degree of substitution (DS) values ranging from 0.012 to 0.290, were used to investigate the difference of binding affinity with *N*-acetyl-phenylalanine at different pH condition. The report showed that their complex at pH 7 was more stable than one at pH 3 as a result of comparative study for binding constants (K_b) though the difference was not great (100 ± 12 at pH 3 and 189 ± 6 at pH 7). However, in the present study, the value of K_b was calculated to be 191.57 ± 13 for pH 3.4, which is 13 times higher than the corresponding value for pH 9.2 (15.27 ± 4).

In this study, we investigated that the synthesis of S-Cys through a one-step chemical modification of Cys isolated from *S. trifolii* TA-1 and its application for pH-dependent inclusion complexation with 4'-hydroxyflavanone. The complex at pH 3.4 was more stable than one at pH 9.2 as a result of comparative study for binding constants. This pH difference of binding constants suggests that molecular complexes of the S-Cys with 4'-hydroxyflavanone are critically dependent of surrounding pH due to the charge distribution of succinyl moieties attached to the S-Cys. As succinyl groups attached to S-Cys act as weak acids with the pK_a value ranging from 4.2 to 5.6, almost all of the succinyl groups of S-Cys at pH 9.2 contain the negative charges which may disturb the effective complexation of S-Cys with 4'-hydroxyflavanone. Thus, very weak binding constant was observed at pH 9.2 compared with acidic conditions at pH 3.4. Throughout the study, pH-dependent differential binding strength of S-Cys was clearly observed, suggesting that S-Cys shows the potential capacity as a novel host chemical modulator responding to the surrounding pH changes.

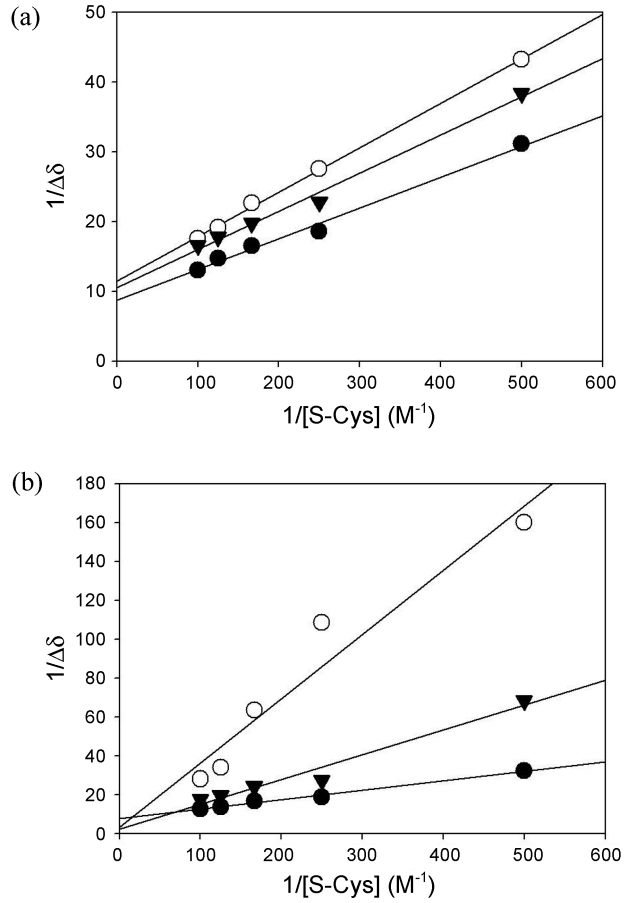

Figure 3. Benesi-Hildebrand plots for 4'-hydroxyflavanone interacted with S-Cys at pH 3.4 (a) and 9.2 (b). Symbols; H5(●), H2' (○) and H8 (▼).

Table 1. Binding constants K_b of 4'-hydrxoyflavanone with S-Cys in acetate and borate buffer (pH 3.4 and 9.2)

Protons	pH = 3.4		pH=9.2	
	K_b	K_{av}	K_b	K_{av}
H5	198.57		16.11	
H2'	180.83	191.57±13.35	10.68	15.27±4.24
H8	195.32		19.04	

Experimental Section

Materials. 2-(4-Hydroxyphenyl)-2,3-dihydro-4*H*-chromen-4-one (4'-hydroxyflavanone; OHFL), succinic anhydride and 4-(Dimethylamino)pyridine (DMAP) were purchased from Aldrich (USA). D_2O (99.9 at.% D) and CD_3OD (40 wt % D) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

Preparation of Cyclosophoraoses (Cys). The *Sinorhizobium trifolii* TA-1 was grown in 500 mL of GMS medium at 30 °C for 12 days. Then, for the isolation, purification and structural analyses of the neutral cyclosophoraoses from the S. *trifolii* TA-1, we followed procedures described elsewhere.[19] Cells were removed by centrifugation at 8000 rpm for 10 min, and then culture supernatants were concentrated five-fold by rotary evaporation. Next, to remove high-molecular-weight (HMW) glycan, the concentrated sample was precipitated by adding 3 volume of ethanol. The HMW compounds were then removed from the concentrated sample by centrifugation at 12,000 rpm for 10 min. Also, the supernatant was concentrated five-fold by rotary evaporation, and in order to get the cyclosophoraoses in a concentrated sample, the product was collected by adding 7 volume of ethanol. After centrifugation, the precipitates were applied to a Bio-Gel P-6 column (2 × 110 cm). The fractions were assayed for carbohydrate by the phenol-sulfuric acid method.[20] The fractions that contained cyclosophoraoses were pooled, concentrated, and desalted by a Sephadex G-10 column (2 × 20 cm). We then applied the desalted sample to a column (2 × 20 cm) of DEAE-cellulose to separate the neutral and anionic cyclosophoraoses. After desalting the cyclosophoraoses with a Sephadex G-10 column (2 × 20 cm), we confirmed them on thin layer chromatography (TLC, isopropanol: water: ethylacetic acid: ammonium water = 5:3:1:1, v/v), NMR spectroscopy and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry.

Preparation of Succinylated Cyclsophoraoses (S-Cys) from Cys. Typically, 500 mg of neutral cyclosophoraoses (0.16 mmol; 3119) was dissolved in 5 mL of anhydrous pyridine. 1 g of succinic anhydride (SA) (10 mmol) was solubilized in 3 mL of pyridine. The solutions were mixed, the mixture was heated at 100 °C and 5 mg of DMAP (4-(dimethylamino)pyridine) (0.041 mmol) were added. The mixture was kept at this temperature for 24 hours. The mixture was evaporated for remove solvent and added 10 mL water. The S-Cys was then precipitated by addition to 50 mL of isopropyl alcohol. The precipitate was washed three times with 10 mL of isopropyl alcohol and finally dried from acetone. The S-Cys was finally purified DEAE sephadex and Bio-gel P2 for removing of remaining chemicals.

Preparation of Inclusion Complexes of the S-Cys with 4'-Hydroxyflavanone. At all the experiments, we made the stock slolution of 4'-hydroxyflavanone from methanol and then diluted the solution with acetate (pH = 3.4) and borate buffer (pH = 9.2) stirring for 24 h in a thermostatic water bath at constant temperature. To determine the binding constants, we dissolved 4'-hydroxyflavanone in acetate and borate buffer that contained 10% methanol with pH 3.4 and 9.2, respectively. The concentration of 4'-hydroxyflavanone was kept constant at 1 mM during the ^{1}H NMR titration experiment. The 4'-hydroxyflavanone was then added to the CM-Cys solutions with concentrations varying from 1 to 10 mM dissolved in acetate and borate buffer that contained 10% methanol, with pH 3.4 and 9.2. Next, we stirred the mixtures for 24 h under darkness, and then degassing them before taking the NMR measurements. We measured the chemical shift of proton and carbon of the S-Cys, 4'-hydroxyflavanone and their complexes. The binding constants were calculated on the basis of Scotts modification[21] of Benesi-Hindebrand equation.[18]

$$\frac{1}{\Delta\delta_{obs}} = \frac{1}{K_b[S-Cys]_T\Delta\delta_{max}} + \frac{1}{\Delta\delta_{max}}$$

In Scotts equation, where $\Delta\delta_{obs}$ is the observed chemical shift difference between the free and complexed guest, $\Delta\delta_{max}$ is the difference in chemical shift between the free and completely complexed guest and $[S\text{-}Cys]_T$ is S-Cys concentrations. The non-linear procedure is an iterative method which has to be started with approximated initial parameters of $\Delta\delta_{max}$ and K_b. In order to obtain these initial parameters, the equation which corresponds to the Benesi-Hildebrand method for a 1:1 stoichiometry,[22] was employed to obtain K_b and $\Delta\delta_{max}$ averages.

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