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Solubility Enhancement of Flavonoids by Cyclosophoraose Isolated from *Rhizobium meliloti* 2011

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Abstract Cyclosophoraose (cyclic β-(1,2)-glucan, Cys) isolated from *Rhizobium meliloti*, a soil microorganism, was used as a solubility enhancer for flavonoids. The complexes of the cyclic oligosaccharide with flavonoids were confirmed through ^{1}H nuclear magnetic resonance (NMR) spectroscopic analysis. Flavonoids solubilized by Cys were quantitatively analyzed through high-performance liquid chromatography (HPLC). Among the flavonoids tested, the solubility of naringenin was greatly enhanced by Cys, compared with other compounds. The solubility of naringenin was enhanced about 7.1-fold by adding 10 mM Cys, compared with a control. ^{1}H NMR spectroscopic analysis indicated that the H-6 and H-8 protons, which are located on the A ring of naringenin, were greatly shifted upfield upon the complexation with Cys. This result suggested that Cys showed a regioselective interaction with the naringenin molecule upon the complexation, resulting in the solubility enhancement of naringenin.

Key words: *R. meliloti*, cyclosophoraose, solubility enhancer, flavonoid

Cys is a class of unbranched cyclic oligosaccharides composed of β-(1→2)-D-glucan as a neutral or anionic form [1]. Cys has been reported to play an important role in regulation of osmolarity in response to external osmotic shock [17] as well as in the successful root-nodule formation of *Rhizobium* species [2]. In relation to the application as a solubility enhancer, many studies have recently been carried out on the complexation of a family of Cys with poorly soluble compounds such as ergosterol, indomethacin, paclitaxel, *N*-acetyltryptophan, and hydrobenzoin [8, 10, 11, 13]. Enantio-differentiating abilities of both neutral and anionic Cys have also been reported, based on a unique interaction [7, 13, 14]. These abilities of Cys could be caused by flexible backbone structures, as proposed in Fig. 1A, and narrower cavity sizes than those expected from their bulky ring sizes [4, 16, 18]. The capacity of Cys to form the complexes suggests that the natural cyclic oligosaccharide may find various applications similar to those currently being studied for cyclodextrins (CDs), which are cyclic oligosaccharides with six (α-CD), seven (β-CD), and eight (γ-CD) glucopyranose residues linked by α-(1,4) glycosidic bonds, synthesized

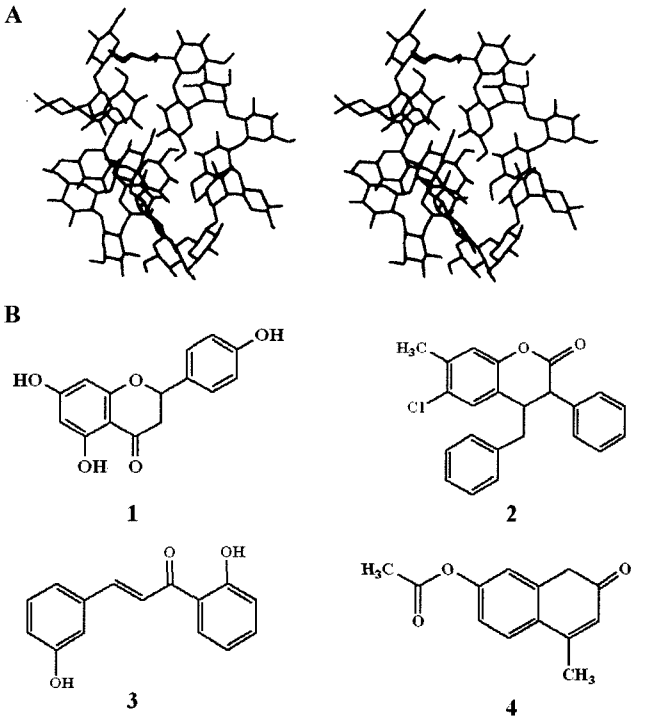

Fig. 1. Stereoview of molecular model of Cys21 [4] (**A**) and chemical structures of the flavonoids (**B**); naringenin (1), 4-benzyl-6-chloro-7-methyl-3-phenylcoumarin (2), 3,2-dihydroxychalcone (3), 7-acetoxy-4-methylcoumarin (4).

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from starch by CD glucanotransferase derived from more than 30 bacteria containing *Bacillus macerans* [9, 20].

Our previous study showed that the solubility of luteolin, a plant flavonoid and *nod* gene inducer [21], was greatly enhanced by adding Cys in water [12]. In the present study, based on high affinity of Cys with the flavonoid molecule, we investigated solubility changes of various flavonoids in the absence and presence of neutral Cys isolated from *R. meliloti* 2011, as a solubilizer [19] and host molecule. As shown in Fig. 1B, naringenin, 4-benzyl-6-chloro-7-methyl-3-phenylcoumarin, 3,2-dihydroxychalcone, and 7-acetoxy-4-methylcoumarin were used as target guest molecules. The extents of complex formation were confirmed at a molecular level by ^{1}H NMR experiment, and concentrations of flavonoids complexed with Cys were then quantitatively determined by HPLC.

R. meliloti 2011 was used to produce cyclosophoraose. Preculture was prepared by inoculating the organism into standard GMS medium [3]. Cells from the late exponential phase were inoculated into a 5-l jar fermentor at 150 rpm for 3 days at 25°C and pH 7.0. Cells were separated from the culture supernatant by centrifugation at 3,000 ×*g* for 20 min at 4°C. Cell pellets were extracted in 75% (v/v) ethanol at 70°C for 30 min, as described previously [3]. After centrifugation, the supernatant was concentrated and loaded onto a Sephadex G-50 column (3×200 cm). Elution was done with water at a rate of 1 ml/min. Fractions were assayed for total carbohydrate by the phenol-sulfuric acid method. The glucan fraction was pooled, concentrated, and desalted by using a Sephadex G-15 column (2×27 cm). Neutral and charged glucans were separated on a DEAE-cellulose column (2×35 cm) by using a gradient of 0 to 200 mM KCl in 10 mM Tris-HCl (pH 8.4). The neutral cyclic glucan fractions were desalted by using a Sephadex G-15 column.

For the phase solubility experiment, guest molecules were first dissolved in MeOH or CH_3CN to obtain 10 mM stock solutions. One ml of the stock solutions was added to 1 ml of various concentrations of aqueous host solution. The mixture was stirred for 24 h at 30°C in the dark. After 24 h, the mixture was partially evaporated, lyophilized, and dissolved in 1 ml of H_2O to remove uncomplexed guest molecules by filtration with a 0.2 μm membrane filter (Whatman BioScience, U.K.). The filtrates were lyophilized and dissolved in mobile phase for HPLC. The Bondclone C18 column (10 μ, 300×3.90 mm) was used for HPLC (Shimadzu, Japan) experiments. The analysis was carried out at 30°C and at the flow rate of 0.8 ml/min. For HPLC analysis of naringenin, 3,2-dihydroxychalcone, and 7-acetoxy-4-methylcoumarin, MeOH-H_2O (60:40, v/v) was applied as a mobile phase, whereas CH_3CN-H_2O (60:40, v/v) was used for 4-benzyl-6-chloro-7-methyl-3-phenylcoumarin. Elution was monitored at 254 nm for 3,2-dihydroxychalcone, 274 nm for 7-acetoxy-4-methylcoumarin, 286 nm for naringenin, and 258 nm for 4-benzyl-6-chloro-7-methyl-3-phenylcoumarin.

For identification of the complexes, NMR spectroscopic analysis was performed on a Bruker (AMX, Germany) spectrometer (500 MHz) with D_2O-CD_3OD (50:50) as a solvent. Tetramethylsilane (TMS, 1%) was used as an internal standard. The ^{1}H spectrum was obtained for the complexes of 10 mM naringenin with an equimolar of Cys and β-CD as host molecules.

Isolated neutral Cys was structurally confirmed, as described previously [8, 10, 11]. Purified native Cys showed an R_f value of 0.125 on TLC. Ring sizes of native Cys were confirmed through MALDI-TOF mass spectrometry, ranging from DP 17 to 27 [8].

Figure 2A shows the phase solubility diagrams of the flavonoids complexed with Cys at 30°C. In the cases of 7-acetoxy-4-methylcoumarin, 3,2-dihydroxychalcone, and 4-benzyl-6-chloro-7-methyl-3-phenylcoumarin, the solubility

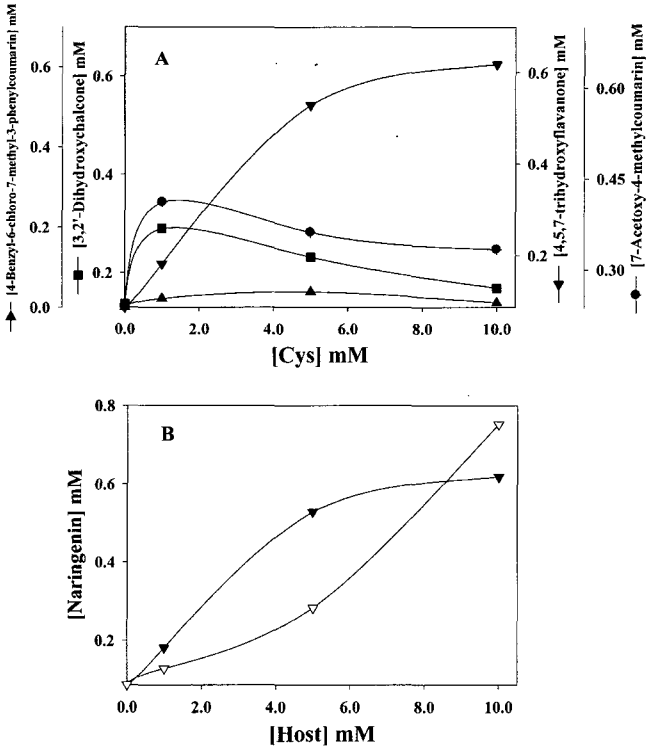

Fig. 2. Phase solubility diagrams of naringenin (▼), 4-benzyl-6-chloro-7-methyl-3-phenylcoumarin (▲), 3,2-dihydroxychalcone (■), and 7-acetoxy-4-methylcoumarin (●) complexed with Cys (**A**), and phase solubility diagrams of naringenin as a function of Cys (▼) and β-CD (▽) concentrations (**B**).

The flavonoids were first dissolved in MeOH or CH_3CN to obtain 10 mM stock solutions. One ml of the stock solutions was added to 1 ml of aqueous host solution with various concentrations. The analysis was carried out at 30°C and at flow rate of 0.8 ml/min. For HPLC analysis of naringenin, 3,2-dihydroxychalcone, and 7-acetoxy-4-methylcoumarin, MeOH-H_2O (60:40, v/v) was applied as a mobile phase, and CH_3CN-H_2O (60:40, v/v) for 4-benzyl-6-chloro-7-methyl-3-phenylcoumarin. Elution was monitored at 254 nm for 3,2-dihydroxychalcone, 274 nm for 7-acetoxy-4-methylcoumarin, 286 nm for naringenin, and 258 nm for 4-benzyl-6-chloro-7-methyl-3-phenylcoumarin.

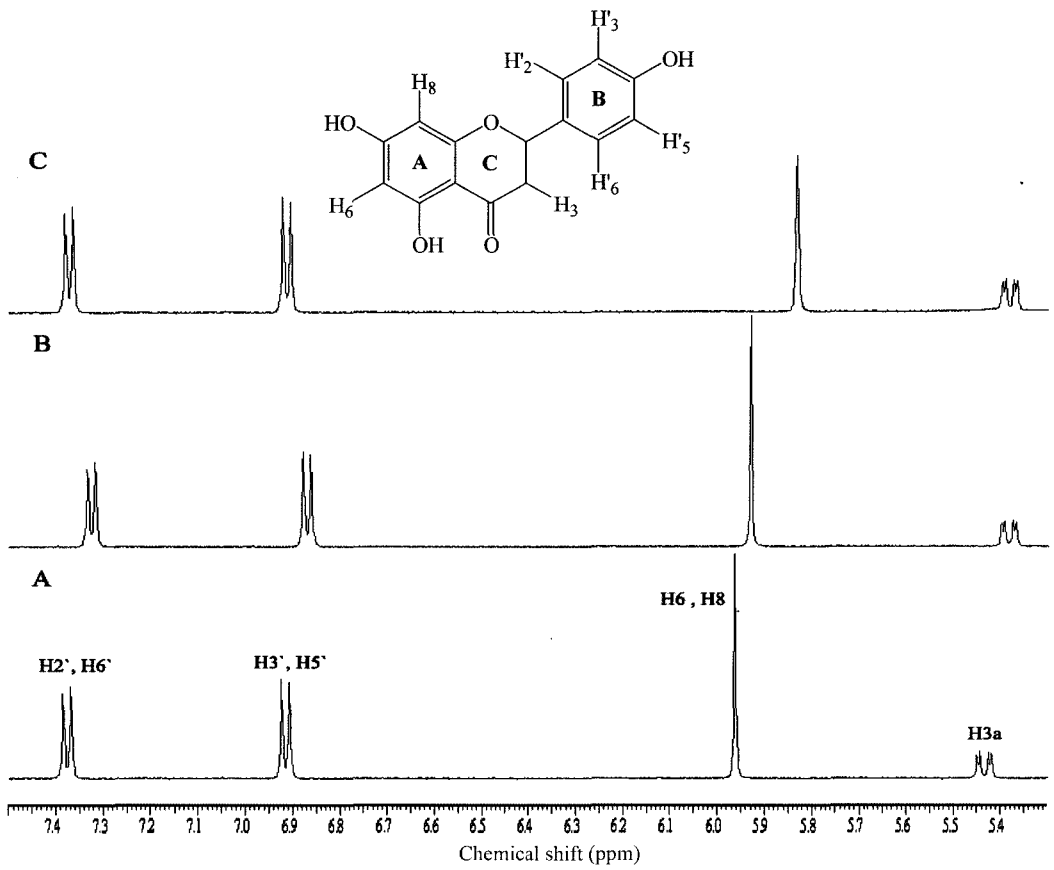

Fig. 3. Partial ^{1}H NMR spectra in the absence or presence of Cys and β-CD: free naringenin (**A**); complex with β-CD (**B**); complex with Cys (**C**). Top structure indicates a numbered structure of naringenin.
^{1}H NMR spectra were obtained for the complexes of 10 mM naringenin with an equimolar of Cys and β-CD as host molecules in D_2O-CD_3OD (50:50) as a solvent. Tetramethylsilane (TMS, 1%) was used as an internal standard.

diagrams measured were typical B_S type, in which the slope was rising in the initial stage and then slightly decreased because of the microcrystalline complex precipitation [6], whereas the diagram for the complex of Cys with naringenin was typical A_N type, in which the slope was rising in the initial stage, followed by a plateau region [6]. The solubilities of the flavonoids tested were enhanced by adding Cys, in order of naringenin, 7-acetoxy-4-methylcoumarin, 3,2-dihydroxychalcone, and 4-benzyl-6-chloro-7-methyl-3-phenylcoumarin. Among them, naringenin showed the highest solubility enhancement with increasing Cys concentration. At the final concentration, the solubility of naringenin was enhanced about 7.1-fold by Cys compared with a control. Figure 2B shows the phase solubility diagrams of naringenin measured by adding Cys or β-CD as a commercial complexing agent in the same condition. The solubility of naringenin was enhanced in a similar extent by the two cyclic oligosaccharides. However, in the complex with β-CD, the diagram showed typical A_P type, indicating different binding patterns of the two host molecules toward the naringenin molecule in aqueous solution.

^{1}H NMR experiment was performed to measure chemical shifts of naringenin by Cys or β-CD upon the complexation. All the protons of naringenin were shifted upfield by adding the two host molecules (Fig. 3 and Table 1). The chemical shifts indicate that the protons of naringenin were surrounded by an electron density of Cys or β-CD upon the complexation. This phenomenon has often been observed in typical inclusion complexation study [5, 12]. Among the protons of naringenin, the H6 and H8 were more affected by Cys than other protons. However, Cys showed a structurally different binding mode toward naringenin in aqueous state, compared with β-CD. Specifically, Cys showed more favorable accessibility for the A ring of naringenin than other ring moieties upon the complexation, whereas β-CD for the B ring.

In this study, we investigated the solubility changes of some flavonoid such as naringenin in the absence or presence of Cys, a natural cyclic oligosaccharide. Among the four flavonoids tested, Cys preferentially enhanced the solubility of naringenin about 7.1-fold, compared with a control. The solubility enhanced by Cys was further confirmed by ^{1}H NMR chemical shift changes upon the complexation.

Table 1. ^{1}H NMR chemical shifts of all the protons of naringenin (refer to the numbered structure in Fig. 3) upon the complexation with Cys or β-CD.

Protons	Naringenin	β-CD		Cys	
	δ^a	δ^b	$\Delta\delta^c$	δ^b	$\Delta\delta^c$
H'2, H'6	7.376	7.325	-0.051	7.370	-0.005
H'3, H'5	6.914	6.869	-0.045	6.911	-0.003
H6, H8	5.961	5.928	-0.033	5.829	-0.132
H3a	5.434	5.382	-0.052	5.379	-0.055
H2	3.193	3.135	-0.058	3.137	-0.056
H3b	2.822	2.782	-0.039	2.759	-0.062

[a]Free state.
[b]Complex state.
[c]$\Delta\delta=\delta^b-\delta^a$.

Through ^{1}H NMR spectroscopic analysis, it was then found that Cys regioselectively complexed with the naringenin molecule, where the A ring moiety of the flavanone was subjected to a specific interaction with the cyclic oligosaccharide. Considering the solubility enhanced by a commercially available β-CD in this study, Cys could function as a good solubilizer for flavonoids and their derivatives. If Cys modified with charged substituents could be made from a neutral form, its complex-forming ability would be expected to be better than native Cys. Further research is in progress.

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