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Solubility Enhancement of Salicylic Acid by Complexation with Succinoglycan Monomers Isolated from *Sinorhizobium meliloti*

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The nitrogen fixation soil bacteria *Sinorhizobium meliloti* (Rm 1021, *S. meliloti*) produce acidic exopolysaccharide (EPS), which may play a crucial role in the development of the root nodule symbiosis between bacterium and legumes of Alfalfa.[1,3,5,6] Succinoglycan octasaccharides secreted by *S. meliloti* consists of a β-1,3, β-1,4, and β-1,6 linked octasaccharide subunit containing one galactose at the reducing end and seven glucose residues, one to two succinyl group located at the C-6 position of the sixth and seventh sugar residue and a pyruvyl group linked to the eighth sugar residue through a 4,6-ketal linkage (Fig. 1).[5,17] In addition, the succinoglycan monomer has been investigated as chiral additives for the separation of chiral flavonoids.[11,12] The successful enantio separation of flavonoids using the succinoglycan monomer is based on the different interactions between the monomer and the R or S configuration of chiral flavonoids. Also, the structural property of the succinoglycan dimers was investigated.[13] In this study, the hydrophobicity of succinoglycan dimers was evaluated based on interactions with hydrophobic fluorescent probes.

Salicylic acid is phenolic phytohormone which has important roles in plant growth and development. Salicylic acid is involved in endogenous signaling it helped in plant defense against pathogens. Salicylic acid acts as systemic acquired resistance (SAR) in which a pathogenic attack on one part of the plant induces resistance in other parts.[9] Furthermore the involvement of rhizobium synthesized Nod factors in the inhibition of salicylic acid mediated defense in legume.[15] Also salicylic acid is known for its ability to ease aches and pains and reduce fevers, those medicinal properties have been widely known since ancient times, and it was used as an anti-inflammatory drug.[16] Recently, salicylic acid and its derivatives are used as constituents of some rubefacient products. For example, methyl salicylate is used as a liniment to soothe joint and muscle pain, and choline salicylate is used topically to relieve the pain of aphthous ulcers. In addition salicylic acid is used as an important ingredient in many skin-care products for the treatment of acne, psoriasis, calluses, corns, keratosis pilaris, and warts.[18] However salicylic acid is poorly soluble in water and also cause irritation of mucus membranes and affects gastrointestinal blood circulation.[2]

Herein highly water soluble succinoglycan monomers were used as solubilizer to increase salicylic acid's solubility. The phase solubility diagram confirmed that its ability to increase solubility of Salicylic acid. The NMR spectroscopy, differential scanning calorimetry (DSC) and Fourier Trans-

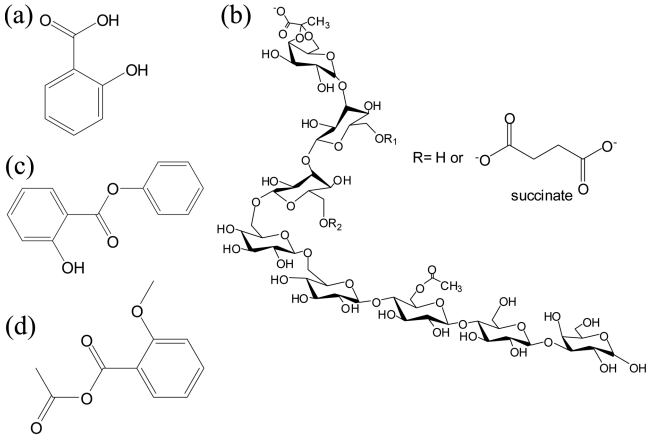

Figure 1. Structure of (a) salicylic acid, (b) the succinogylcan monomer, where R_1 and R_2 indicate the sites for the succinyl group (c) phenyl salicylate and (d) methyl acetyl salicylate.

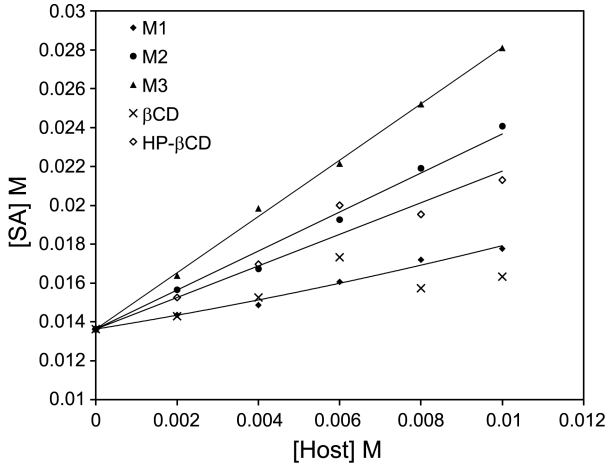

Figure 2. Phase solubility diagram of salicylic acid at various concentrations (0 to 10 mM) of M1 (◆), M2 (●), M3 (▲), β-CD (X), and HP β-CD (◇) complexes in water at 30 °C.

form Infrared Spectroscopy (FT-IR) assays shows that succinoglycan octasaccharides forms complex with salicylic acid successfully. Our results suggest that succinoglycan monomers can form efficient complex with salicylic acid than its derivates.

The phase solubility diagram of salicylic acid/succinoglycan monomers (M1, M2, and M3), β-CD and 2-hydropropyl-β-cyclodextrin were investigated. Figure 2 shows the A_L type diagram for the complex of succinoglycan monomers with salicylic acid is indicating that the complexes are of first order with respect to succinoglycan monomers. However, the phase solubility diagram for both M2 and M3 shows the slopes greater than 1 indicating that the complexes are of second or higher order with respect to the drug.[8] The binding constants, K, of the complexes were calculated using equation (1) and (2).[8] The binding constant for 1:1 M1/salicylic acid is determined to $K_{1:1} = 17\ M^{-1}$. And the binding constants for the 2:1 M2/salicylic acid and M3/salicylic acid are $K_{2:1} = 5{,}415\ M^{-2}$ and $K_{2:1} = 1{,}729{,}735\ M^{-2}$ respectably.

The thermal properties of the monomers, salicylic acid, and complexes were investigated by DSC (Figure 3). The DSC curves of the raw materials (monomers and salicylic acid) were compared with monomers/salicylic acid complex. Successful formation of complex was confirmed by the absence of the melting endothermic peak of salicylic acid at 159 °C.

The physical property of complex was also detected by FT-IR spectra. The partial FT-IR spectra of salicylic acid, M3, their physical mixture, and their complex are shown in Figure 4. The spectrum of salicylic acid contained the characteristic absorption band for carbonyl stretching vibration at 1656, aromatic C=C stretching at 1482, and 1444 cm^{-1} and *o*-disubstituion of salicylic acid at 757 (Figure 4(a)), whereas the M3 spectrum contained carbonyl stretching vibration bands at 1733 and 1621. The FT-IR spectrum of the physical mixture contained absorption peaks with reduced intensity at the same position as the both aromatic C=C stretching vibration and *o*-disubstitution of salicylic acid, which likely resulted from the addition of salicylic acid and M3. However, those characteristic absorption peaks disappeared in the complex products, suggesting that the drug environment was modified.

The NMR spectra were used to elucidate in detail the structures of the complexes of succinoglycan monomer with salicylic acid in aqueous solution. The assignments of the proton signals of salicylic acid are shown in Figure 5, and the peaks were shifted by the addition of succinoglycan monomers. All protons from salicylic acid were upshifted. Table 1shows the variations in the chemical shifts of salicylic acid by succinoglycan monomers. Table 1 shows chemical shifts for the complexes of succinoglycan monomers with salicylic acid.

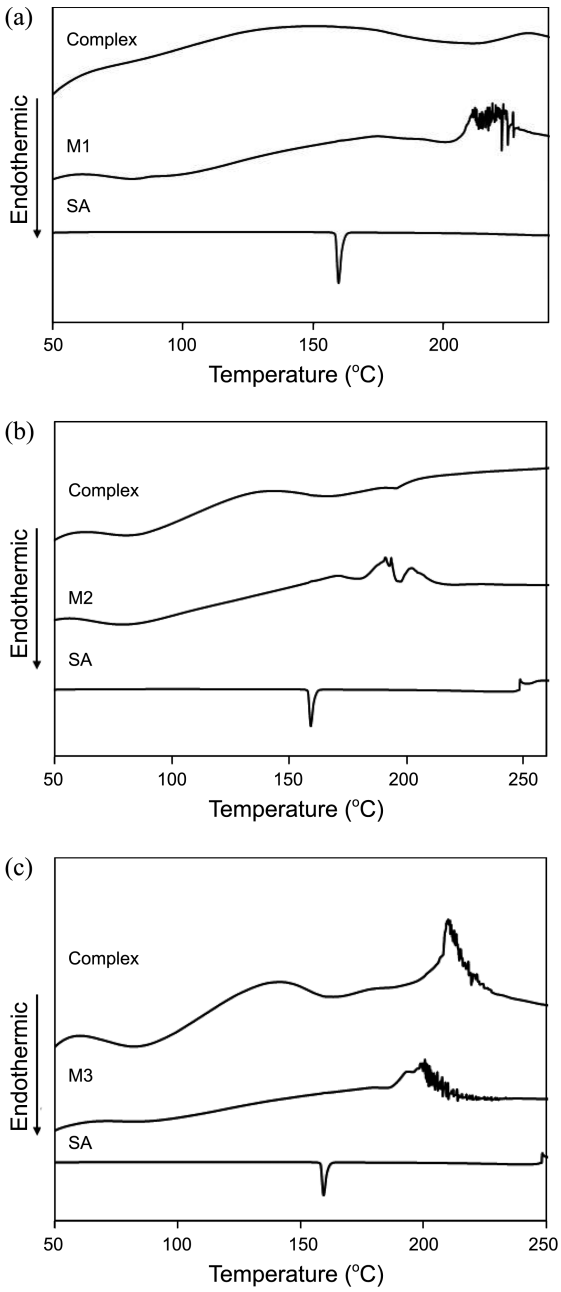

Figure 3. DSC curves of salicylic acid, succinoglycan monomer, and equmolar complexes (10 mM) (a) M1, (b) M2, and (c) M3.

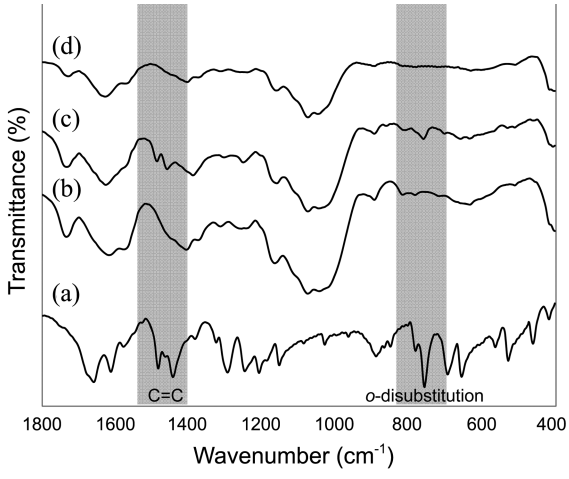

Figure 4. Partial FT-IR spectra of (a) Salicylic acid, (b) M3, (c) Physical mixture and (d) salicylic acid/M3 complex. Spectra were acquired between 4000 and 400 cm^{-1}.

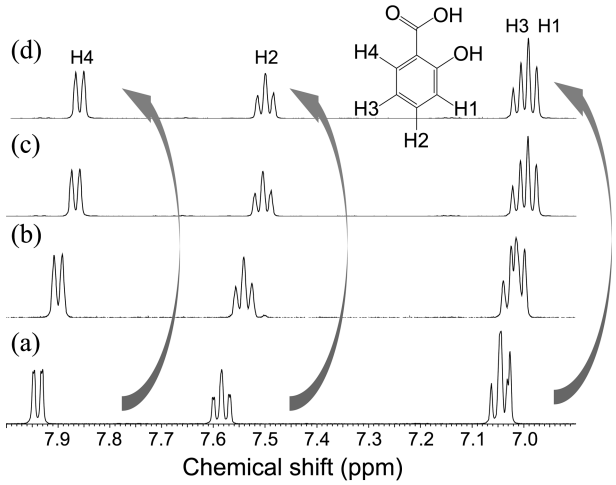

Figure 5. The partial ^{1}H NMR spectra of (a) salicylic acid, equimolar complex of (b) M1/Salicylic acid, (c) M2/Salicylic acid and (d) M3/salicylic acid in a solution of D_2O (D, 99.96%).

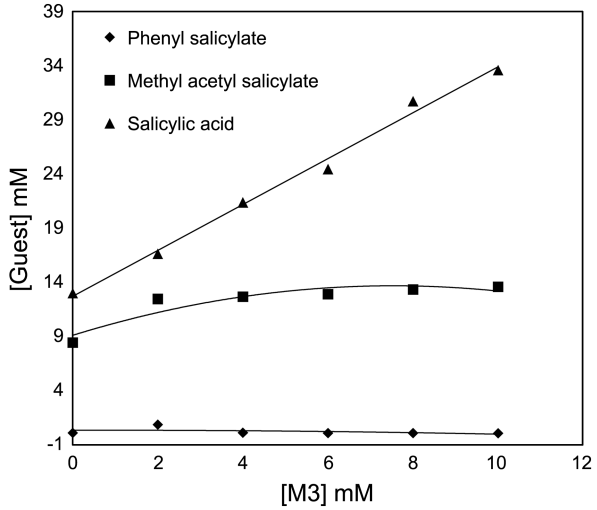

Figure 6. The phase solubility diagram of (a) salicylic acid, (b) Methyl acetyl salicylic acid, and (c) Phenylsalicylic acid complexed with various concentration (0 to 10 mM) of M3.

The succinoglycan monomer M3 shows the binding specificity with salicylic acid comparing with its chemical derivatives. The solubility of complex between succinoglycan monomer M3 and phenyl salicylate (Figure 1(c)) or methyl acetyl salicylate (Figure 1(d)) was investigated. As shown in Figure 6, the phase solubility diagram showed A_L type slope for the complex of M3/salicylic acid whereas B_S type slope was observed for the complex of both M3/phenyl salicylate and M3/methyl acetyl salicylate. B_S type response denotes complexes of limited solubility.[14] Previously, the involvement of salicylic acid in the establishment of the *S. meliloti* and alfalfa symbiosis was investigated by Mrtines-Abarca *et al*. (1998),[15] they suggested that Nod factors controls the suppression of the salicylic acid accumulation. Also, *Rhizobium* produces unidentified elicitors that induce salicylic acid accumulation in alfalfa. The initial interaction between the two symbiotic partners is a highly specialized process and involves an exchange of signals between the host plant and the colonizing bacteria.[7] For these respect, succinoglycan and salicylic acid may form more effective complex compared to salicylic acid derivates complex.

Table 1. Chemical shift of succinoglycan monomers and salicylic acid complexes

	Proton of Salicylic acid	δ (free)	δ (complex)	Δδ
M1	H1	7.032	7.016	0.016
	H2	7.601	7.557	0.044
	H3	7.063	7.041	0.022
	H4	7.949	7.908	0.041
M2	H1	7.032	6.993	0.039
	H2	7.601	7.520	0.081
	H3	7.063	7.023	0.040
	H4	7.949	7.874	0.075
M3	H1	7.032	6.985	0.047
	H2	7.601	7.506	0.095
	H3	7.063	7.016	0.047
	H4	7.949	7.861	0.088

In conclusion, complexation of the succinoglycan monomers with salicylic acid was studied using phase solubility diagram. The aqueous solubility of salicylic acid was enhanced by the function of succinoglycan monomers concentration. The solubility enhancement effect of M3 was found to be the higher than those of the other succinoglycan monomers (M1, and M2). The DSC and FT-IR analysis also confirmed effective formation of succinoglycan monomer complexes with salicylic acid. Also, the interaction of succinoglycan monomers with salicylic acid was analyzed by ^{1}H NMR spectroscopy. Based on these results, the succinoglycan monomers originated from *S. meliloti* used to enhance the solubility and stability of salicylic acid. The solubility of the complex of succinoglycan monomers with salicylic acid was much improved than previous study of the complex of 2-hydropropyl-β-CD with salicylic acid.[2] Furthermore, high binding constant of succinoglycan monomers and salicylic acid complex may be explained by their symbiotic relationship between the host and its symbiotic bacteria.

More detailed studies on the biological function of succinoglycan monomers in the symbiosis with legume plants are in progress.

Experimental Section

Materials. Salicylic acid (puriss. p.a., 99.0% (T)), phenyl salicylate, β-cyclodextrin and 2-hydropropy-β-cyclodextrin (HP-β-CD) were purchased from Sigma-Aldrich Chemials Co. (St. Louis, MO, USA). Methyl acetyl salicylate was purchased from Tokyo Chemical Industry Co., LTD. (Tokyo, Japan) D_2O (99.9 at.% D) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). They were employed without further purification.

Preparation of Succinoglycan Monomers. The isolation and purification of succinoglycan monomers from *S. meliloti*

were carried out as described previously.[19] *S. meliloti* was cultured in 500 mL of GMS medium at 30 °C and 150 rpm for 5 days. Cells were removed by centrifugation (8000 × g for 10 min). After centrifugation, the supernatant was concentrated by a factor of five relative to the original volume by rotary evaporation. The supernatant contained exopoly saccharide (EPS) and EPS was precipitated from the concentrated supernatant by adding three volumes of ice-cold ethanol and then the sample was subjected to centrifugation (8000 × g for 10 min). After removing the EPS, the supernatant was again concentrated by a factor of five relative to its original volume. Succinoglycan was then precipitated by adding another seven volumes of ice-cold ethanol and then subjecting the sample to centrifugation. The precipitate was dissolved in distilled water. Chromatography was the performed for further purification. Samples were applied to a column of Bio-Gel P6 (2.5 × 145 cm), which was eluted at room temperature with 0.5% AcOH. The monomer fraction was pooled and concentrated. The concentrated sample was separated into three fractions (M1, M2, and M3) using DEAE Sephadex A-25 (1.5 × 48 cm), according to succinate moiety (Figure 1). The samples were eluted with KCl in an MOPS buffer using linear gradients of 5-250 mM KCl. Fractions were collected and subsequently desalted using a Bio-Gel P2 (2 × 48 cm) column. The purified succinoglycan monomers were confirmed through MALDI-TOF mass spectrometry (Voyager-DETM STR Bio-Spectrometry, Applied Biosystems, Framingham, MA, USA) in the negative ion mode using 2,5-dihydroxybenzoic acid (DHB) as the matrix.[10] The mass spectra were recorded in DHB at a molar ratio of 10^{-3} with a total loading of around 1 μg of sample.

Phase Solubility Analysis. Phase solubility study of succinoglycan monomers and salicylic acid were performed according to Higuchi and Connors method. The excess amount of Salicylic acid (50 mM) was added to unbuffered aqueous solutions of succinoglycan monomers (M1, M2 and M3) (0.0-10.0 mM) in capped vials, then sonicated for 10 min. Vials were sealed to avoid changes due to evaporation and magnetically stirred for 24 h at 30 °C, shielded from light to prevent degradation of the molecules. After the equilibrium, the samples were filtered through a PVDF 0.2 μm filter (Whatman). Each sample was analyzed by UV-vis spectrophotometry (UV 2450, Shimadzu Corporation) from 220 to 350 nm at 30 °C to evaluate the concentration of the Salicylic acid dissolved.

Linear phase-solubility diagrams (A_L-type) shows are first order with respect to the succinoglycan monomers and first or higher order with respect to the drug. If the slope of an A_L-type system is greater than one, higher order complexes are indicated. A slope of less than one does not necessarily exclude higher order complexation but 1:1 complexation is usually assumed in the absence of other information. The slope of the linear phase solubility diagram will be determined by the equation:

$$K_{1:1} = \text{slope}/S_0(1-\text{slope}) \quad (1)$$

$$\text{Slope} = 2S^2{}_0 K_{2:1}/S^2{}_0 K_{2:1} + 1 \quad (2)$$

where S_0 free salicylic and K is the binding constant of the complex.

Differential Scanning Calorimetry. The physical properties of the succinoglycan monomers and salicylic acid were measured by Differential scanning calorimetry DSC Q200 V24.4 (TA Instruments, USA). Approximately 5 mg of salicylic acid, monomers, and salicylic acid/monomer complex sample was heated in a sealed aluminum pan, using an empty sealed pan as a reference, over the temperature range of 30 to 300 °C at a rate of 10 °C min^{-1}. An indium standard was used to calibrate the temperature scale.

FT-IR Spectroscopic Analysis. Fourier-transform infrared spectra were obtained on a Bruker IFS-66/Sspectrometer (AMX, Germany). 1.5-2.0 mg of four different samples, salicylic acid, monomers, physical mixture and salicylic acid/monomers complex, were mixed with a KBr pellet.

Nuclear Magnetic Resonance (NMR) Spectroscopy. For the NMR spectroscopic analysis, we used a Bruker Avance 500 spectrometer to record ^{1}H-NMR spectra. The NMR spectroscopic analysis was carried out in D_2O at room temperature.

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