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Hydrophobic Interactions of Succinoglycan Dimers Isolated from *Sinorhizobium meliloti* with Hydrophobic Fluorescence Probes, 8-anilino-1-naphthalenesulfonate and 6-*p*-toluidino-2-naphthalenesulfonate

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Succinoglycan, an acidic exopolysaccharide, is produced by the nitrogen-fixing bacterium, *Shinorhizobium meliloti*.[1] The main chain 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. Also, the repeating unit carries pyruvyl, acetyl, and succinyl modifications. The acetyl group is located at the C6 position of the third sugar residue from the reducing galactose, the succinyl group is located at the C6 position of the seventh sugar, and the pyruvyl group is linked to the eighth sugar residue through a 4,6-ketal linkage.[2,3]

The succinoglycan has high molecular weight (HMW) forms consisting of hundreds of octasaccharide units and low molecular weight (LMW) forms that are composed of monomers, dimers, and trimers of the octasaccharide unit. The LMW succinoglycan offers the advantages of the HMW succinoglycan for research because it is not viscous and the structure is simpler than the HMW succinoglycan. Also, the LMW succinoglycan is of particular interest, as some reports have described that it is able to restore the ability of invasion-deficient *S. meliloti* mutants to invade nodules.[4-7]

In addition to investigation of its biological activity, physico-chemical properties of the LMW succinoglycan have been studied. Monomers of succinoglycan have been isolated and investigated as chiral additives in capillary electrophoresis (CE) for the chiral separation of some flavonoids.[8,9] The effect of enantioseparation is known to be dependent on the succinate moiety of the monomer structure. Also, flavonoid enantioseparations using succinoglycan monomer show that the monomer may directly interact with flavonoids produced by the host plant. However, there have been no reports on physico-chemical properties of dimers or trimers. Herein, we examine the structural properties of the dimers. The chemical structures of succinoglycan dimers are also shown in Figure 1(a).

Fluorescent probes are defined as small molecules that undergo changes in one or more of their fluorescent properties as a result of hydrophobic interaction with environmental macromolecules.[10] As representative fluorescent probes, 8-anilino-1-naphthalene sulfonate (ANS) and 6-*p*-toluidino-2-naphthalenesulfonate (TNS) have been used to explore the hydrophobicity of biomolecules.[11-13] The chemical structures of both fluorescent probes are shown in Figure 1(b) and 1(c). In the present study, the fluorescence of ANS and TNS, respectively, in an aqueous solution was investigated under the presence of succinoglycan dimers (D1, D2, D3, and D4), and the hydrophobic character of the dimers was analyzed. Based on double reciprocal plots of the fluorescence increments depending on the dimer concentration, the association constants could be estimated. The results indicate that association constants of ANS and TNS, respectively, with D2 are the largest among the 4 types of

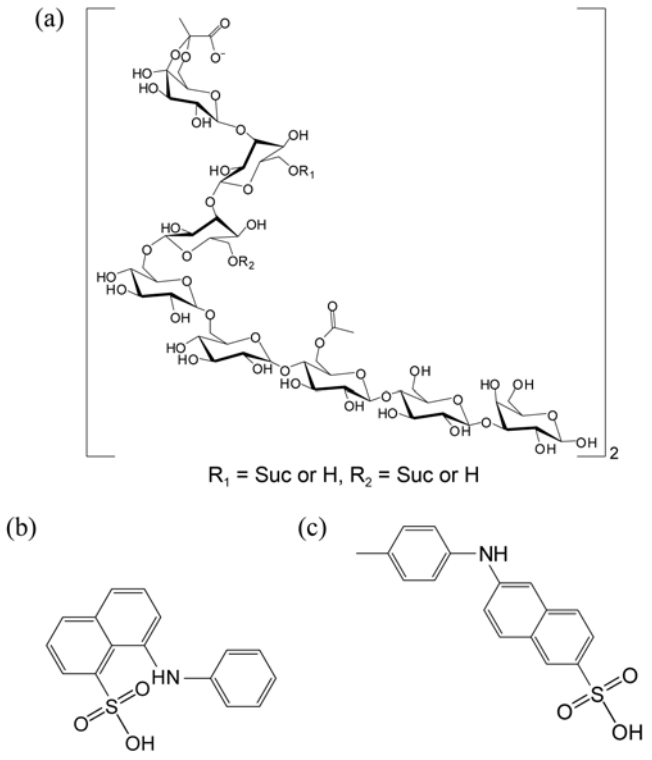

Figure 1. Chemical structures of succinoglycan dimers (a), ANS (b), and TNS (c). The succinoglycan dimers are classified with D1, D2, D3, and D4 according to the number of succinyl groups in the molecule.

dimers. Furthermore, the ANS interacted with D2 was analyzed with two dimensional nuclear magnetic resonance (2D NMR) spectroscopy, and the results provided key evidence for the potential of a hydrophobic nature of succinoglycan dimers containing D2.

S. meliloti Rm 1021 was grown in a GMS medium, and the LMW succinoglycan (monomers, dimers, and trimers) was purified through size exclusion chromatography from the supernatant after removing HMW succinoglycan. As further separation of dimers, anion exchange chromatography through the DEAE Sephadex A-25 was carried out for the fractionazation of D1, D2, D3, and D4, respectively. Finally, each fraction was desalted and lyophilized. The structures of obtained dimers were confirmed with MALDI-TOF MS (matrix assisted laser desorption/ionization - time of flight mass spectrometry) in our report.[14]

To confirm that succinoglycan dimers can interact with ANS and TNS, we utilized fluorescence spectroscopic analysis. The ANS and TNS emission spectra depending on the D2 concentration are presented in Figure 2(a) and 2(b). As well as the enhanced fluorescence intensity of ANS, the emission maximum was blue-shifted to about 430 nm from 515 nm. This blue-shift in the ANS emission along with the increased intensities is due to inclusion of ANS in the hydrophobic area produced by D2.[10,11] The similar increasing pattern was also obtained from TNS with D2 (Figure 2(b)). Previous studies have demonstrated that β-cyclodextrin (β-CD) increases the fluorescence of ANS, since ANS is captured in the nonpolar cavity of CD.[15,16] Thus, these effects of succinoglycan dimers on both fluorescent probes (ANS and TNS) reveal the hydrophobic nature of those molecules, although the specific three-dimensional structure of D2 complexed with the probes has not yet been elucidated.

To obtain association constants from the fluorescence data, we used the following Benesi-Hildebrand equation.[17]

$$1/(F - F_0) = 1/(F_\infty - F_0)K_a[\text{succinoglycan dimer}] + 1/(F - F_0)$$

where F_0 is the fluorescence intensity in the absence of the succinoglycan dimer, F is the fluorescence intensity at a particular concentration of succinoglycan dimer, F_∞ is the fluorescence intensity when all fluorescent probes are complexed, and K_a is the association constant for the 1:1 complex.

Figure 3 shows double reciprocal plots of $1/(F-F_0)$ *vs* 1/[succinoglycan dimer] for ANS and TNS complexed with succinoglycan dimers. The linear plot implies the formation of inclusion complexes with a stoichiometry of 1:1.[11,17] The association constants were determined from the ratio of the intercept to the slope. As given in Table 1, the association constants of D2 with ANS and TNS, respectively, are the largest among the succinoglycan dimers, with values of 464 and 310 M^{-1}, respectively. The K_a value of D2 with ANS is much larger than that (73 M^{-1}) for the complexation of β-CD with ANS (data not shown) indicating that the hydrophobic environment produced by the succinoglycan dimer certainly

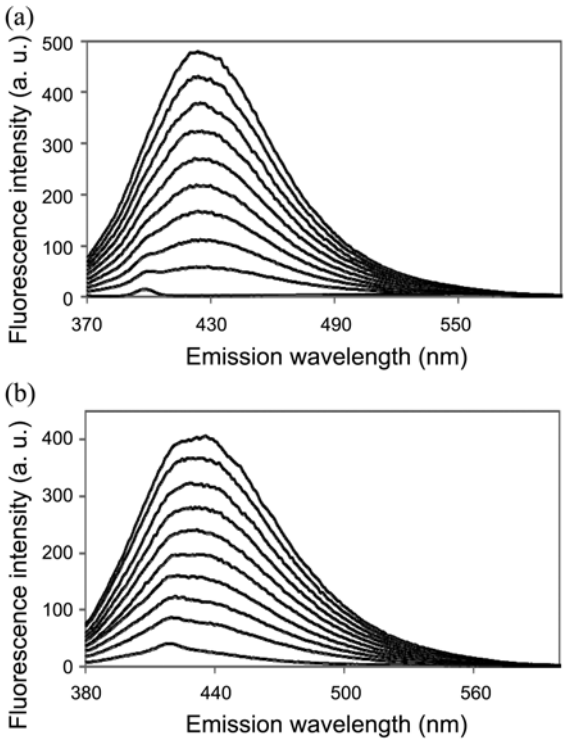

Figure 2. Fluorescence emission spectra of ANS (a) and TNS (b) in various concentrations of D2. [D2] = 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 mM, [ANS] and [TNS] = 2.5 μM.

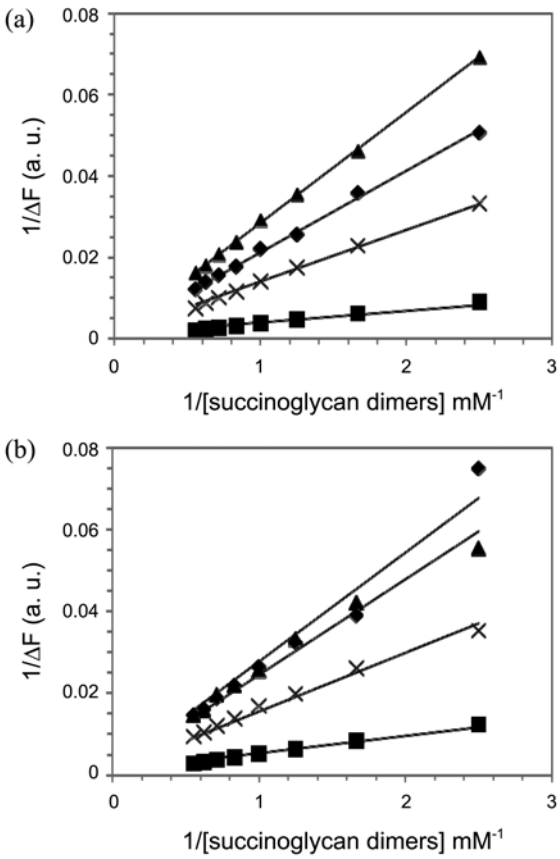

Figure 3. Double reciprocal plot of the concentration of succinoglycan dimers (D1; ◆, D2; ■, D3; ▲, D4; ×) and the increase in fluorescence intensity of ANS (a) and TNS (b).

Table 1. Association constants for ANS/succinoglycan dimers and TNS/succinoglycan dimers

Succinoglycan dimers	ANS K_a (M^{-1})	TNS K_a (M^{-1})	K_{aANS}/K_{aTNS}
D1	65	49	1.3
D2	464	310	1.5
D3	48	56	0.9
D4	102	91	1.1

affects ANS fluorescence. However, the binding affinities of succinoglycan dimers with ANS and TNS are similar as based on the value of K_{aANS}/K_{aTNS}, whereas β-CD binds TNS 18 fold more strongly than ANS.[12] The notable aspect of this result is that the succinoglycan dimer is an acyclic hexadecasaccharide containing 14 glucoses and 2 galactoses substituted with succinyl, acetyl, and pyruvyl moieties and, the dimer has no distinct cavity in its structure. This result might be possible in that linear oligosaccharides can exhibit amphiphilicity caused by hydrophilic and hydrophobic surfaces in sugar chains.[18,19] In the previous study,[12] the complexation ability of maltooligosaccharides (maltopentaose, maltohexaose and maltoheptaose) has been suggested through hydrophobic interaction with ANS or TNS. Although succinoglycan dimers have a backbone structure different from maltooligosaccharides, they also interact with hydrophobic fluorescent probes. To identify the effect of other carbohydrates on the emission spectra of ANS, we investigated their fluorescence intensities. Figure 4 shows the comparative emission spectra of ANS with other carbohydrates which are amylose, β-CD, glucose, and trehalose, respectively. The used amount of other carbohydrates was 3 mg according to 1 mM D2 in 1 mL solution. As another control, the fluorescence by D2 alone is shown as well. This result confirms that the observed fluorescence spectra are characteristic features of fluorescent probes (ANS and TNS) by the succinoglycan dimers.

Furthermore, two dimensional NOE signals of the D2/ANS mixture were detected through a nuclear Overhauser

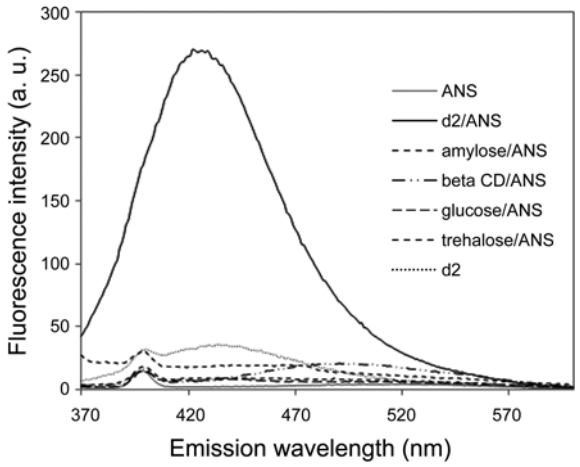

Figure 4. Emission spectra of ANS with other carbohydrates (amylose, β-CD, trehalose, and glucose) and D2 alone.

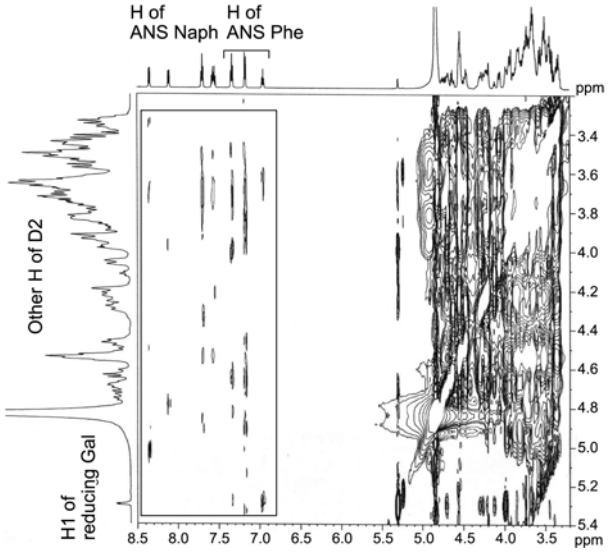

Figure 5. Partial NOESY spectrum of a D_2O solution equimolar in ANS (14 mM) and D2 (14 mM). The rectangle contains the cross-peaks arising from the NOE interactions between protons of D2 and protons of ANS.

enhancement spectroscopy (NOESY) experiment. This approach is frequently used to elucidate the intermolecular interaction of inclusion complexes, since two protons located closely in space can induce an NOE cross-peak.[20] Because most protons of D2 overlapped, forming a broad hump of signals from 3 to 4 ppm except H1 of the reducing sugar, it is not clearly distinguishable which protons of D2 are correlated to protons of ANS. It was determined, however, that protons of ANS are associated with protons of the surrounding D2. Figure 5 illustrates the expanded region of the 2D NOESY spectrum of an ANS/D2 mixture. Intermolecular NOEs between protons of ANS and protons within D2 were observed in combination with intramolecular NOEs by protons in D2. This demonstrates the proximity between the protons of ANS and D2. However, TNS with D2 could not be compared exactly because of low solubility of TNS in D_2O.

In conclusion, we demonstrated whether succinoglycan dimers interact with ANS or TNS through fluorescence spectroscopy. The fluorescence intensities were enhanced by the dimers. This reveals that the structure of linear hexadecasaccharide provides hydrophobicity for ANS molecule. D2 showed the largest effect on the emission spectra, which was also confirmed with NMR spectroscopy. To provide hydrophobic space as a linear carbohydrate, succinoglycan dimers might undergo an induced-fit type adjustment.[12] Based on this study, further interaction studies of succinoglycan dimers as host with other hydrophobic guest compounds can be expected.

Experimental Section

Chemicals. ANS, TNS, amylose, β-CD and glucose were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, Mo, USA). Trehalose was obtained from Fluka Chemical

Co. (St. Gallen, Switzerland).

Bacterial Cultures and the Purification of Dimers. *S. meliloti* Rm 1021 was grown in a rotary shaker at 30 °C in a GMS medium for 5 days.[3] Cells were removed by centrifugation, and the supernatant was concentrated fivefold with rotary evaporation. After adding 3 volumes of ice-cold ethanol, HMW succinoglycan was precipitated. The LMW succinoglycan that remained in the supernatant was concentrated again, and another 7 volumes of ice-cold ethanol were added. The supernatant was collected by centrifugation, and the putative LMW succinoglycan samples were applied to Bio-gel P6 with 0.5% acetic acid. The monomers, dimers, and trimers of the succinoglycan subunit were separated. The dimers were further fractionated to D1, D2, D3, and D4 on a DEAE Sephadex A-25 with a linear gradient from 5 to 400 mM KCl in 10 mM MOPS buffer. Each dimer (D1, D2, D3, and D4) was collected and desalted with a Bio-gel P4 column.

Fluorescence Measurement. The fluorescence of ANS and TNS solutions in the presence of succinoglycan dimers was observed using a spectrofluorophotometer (SIMADZU, RF-5310PC). Measurements were performed in a 0.1 M phosphate buffer (pH 7) at 25 °C. The excitation wavelengths of ANS and TNS were 350 and 366 nm, while the emission wavelength ranged from 370 to 600 nm. The excitation and emission slits had 5 nm widths.

NMR Spectroscopy. For the NMR spectroscopic analysis, we used a Bruker Avance 600 spectrometer to record the NOESY spectrum. The NOESY spectra were recorded with 256/2048 complex data points using a pulse train to achieve a spin-lock field with a mixing time of 800 ms for the complex. The NMR spectroscopic analyses were carried out in D_2O at room temperature.

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References

1. Reuber, T. L.; Walker, G. C. *Cell* **1993**, *74*, 269.
2. Chouly, C.; Colquhuon, I. J.; Jodelet, A.; York, G.; Walker, G. C. *Int. J. Biol. Macromol*. **1995**, *17*, 357.
3. Wang, L.; Wang, Y.; Pellock, B.; Walker, G. C. *J. Bactriol.* **1999**, *181*, 6788.
4. Battisti, L.; Lara, J. C.; Leigh, J. A. *Proc. Natl. Acad. Sci. U. S. A.* **1992**, *89*, 5625.
5. Gonztlez, J. E.; York, G. M.; Walker, G. C. *Gene* **1996**, *179*, 141.
6. Wang, L.; Melean, L. G.; Seeberger, P. H.; Walker, G. C. *Carbohydr. Res*. **2001**, *333*, 73.
7. González, J. E.; Semino, C. E. Wang, L.; Castellano-torres, L. E. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 13477.
8. Kwon, C.; Yoo, K. M.; Jung, S. *Carbohydr. Res.* **2009**, *344*, 1347.
9. Kwon, C.; Paik, S. R.; Jung, S. *Electrophoresis* **2008**, *29*, 4284.
10. Brand, L.; Gohlke, J. R. *Annu. Rev. Biochem*. **1972**, *41*, 843.
11. Nozaki, T.; Maeda, Y.; Ito, K.; Kitano, H. *Macromolecules* **1995**, *28*, 522.
12. Aoyama, Y.; Otsuki, J.; Nagai, Y.; Kobayashi, K.; Toi, H. *Tetrahedron Lett*. **1992**, *33*, 3775.
13. Hirasawa, T.; Maeda, Y.; Kitano, H. *Macromolecules* **1998**, *31*, 4480.
14. Kwon, C.; Lee, S.; Jung, S. *Carbohydr. Res.* **2011**, *346*, 2308.
15. Nishijo, J.; Yasuda, M.; Nagai, M.; Sugiura, M. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 2869.
16. Nishijo, J.; Nagai, M. *J. Pharm. Sci.* **1991**, *80*, 58.
17. Catena, G. C.; Right, F. V. *Anal. Chem.* **1989**, *61*, 905.
18. Balasubramanian, D.; Raman, B.; Sundari, C. S. *J. Am. Chem. Soc.* **1993**, *115*, 74.
19. Das, K.; Sarkar, N.; Das, S.; Bhattacharyya, K.; Balasubramanian, D. *Langmuir* **1995**, *11*, 2410.
20. Ishizuka, Y.; Fujiwara, M.; Kanazawa, K.; Nemoto, T.; Fujita, K.; Nakanishi, H. *Carbohydr. Res*. **2002**, *337*, 1737.