Structural Analyses of Total Anionic Cyclosophoraoses Synthesized by *Rhizobium meliloti* 2011

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Cyclosophoraoses (Figure 1) are unique molecules found almost exclusively in the members of *Rhizobiaceae* family, which are fast growing soil bacteria. These are a class of unbranched cyclic oligosaccharides composed of β-(1→2)-D-glucans varying in size from 17 to 40 as a neutral or anionic form. Recently, many studies have been focused on the inclusion complexation of cyclosophoraoses as the effective solubilizer of various insoluble compounds such as ergosterol, fluoroscein, indomethacin, paclitaxel and vitamins.[2-6] The anionic cyclosophoraoses located in the periplasmic place of the bacteria played important roles on the osmotic regulation as well as on the successful root-nodule formation of *Rhizobium* species at the initial stage of the nitrogen fixation.[7-9] The partial structures of anionic cyclosophoraoses were known to be substituted with charged molecules such as *sn*-glycerol-1-phosphate, succinic acid, and methylmalonic acids,[10-14] depending on the kind of *Rhizobium* species. The structures of anionic cyclosophoraoses were deduced from a fast atom bombardment mass spectrometry (FAB-MS)[13] and NMR spectroscopy.[10,11,15-17] However, these methods did not show the complete structures of exact anionic cyclosophoraoses. Their analyses were restricted to a few isolated fractions of anionic cyclosophoraoses. Moreover, any investigations were not performed on whether all the neutral cyclosophoraoses were to be substituted with anionic moieties or not. So, it still remains unclear whether all the neutral cyclosophoraoses could be substrates for anionic ones.

In this study, we first present the structural analyses of the whole anionic cyclosophoraoses synthesized from *Rhizobium meliloti* 2011 by an electrospray ionization - mass spectrometric (ESI-MS) and two-dimensional NMR spectroscopic methods. Especially, ESI-MS has been widely used in various fields as a high-efficiency and high-resolution separation technique.[18] Recently, highly sulfated cyclic oligosaccharides have been successfully characterized by this technique.[19] Using this ESI-MS techniques, we identified the degree of anionic substitution (DAS) for the anionic cyclosophoraoses produced by *R. meliloti* 2011 in relation with the size distribution. Additional two-dimensional NMR spectroscopic analysis was also used to confirm the exact position of the substituted glycerolphosphates for the isolated anionic cyclosophoraoses from *R. meliloti* 2011.

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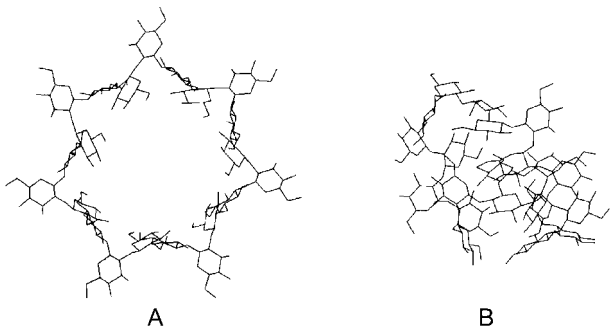

Figure 1. Proposed molecular models of neutral cyclosophoraoses. (A) Palleschi *et al.*[1] (B) Jung *et al.*[2]

Experimental Section

Bacterial strain and culture condition. The bacterial strain used in this study was *Rhizobium meliloti* 2011. It was generously provided by Dr. R. I. Hollingsworth, MSU, E. Lansing, Michigan, U.S.A. For large-scale isolation of cellular glucans, 25 mL preculture was inoculated into 500mL GMS standard medium[20] and cells were cultured to late logarithmic phase and incubated at 30 °C, at 150 rpm on a rotary shaker.

Preparation of cyclosophoraoses. Cells were harvested by centrifugation (8,000 rpm, at 4 °C), washed once with a saline solution, and subjected to the hot-ethanol extraction method. Cells were then extracted with 40 mL of 75% (v/v) ethanol at 70 °C for 30 min. After centrifugation, the supernatant was concentrated on a vacuum rotary evaporator. The concentrated sample was chromatographed on a Sephadex G-50 column (1.5 × 110 cm) at a rate of 20 mL/h and the eluant fractions (7 mL) were assayed for carbohydrate by a phenol-sulfuric acid method. The fractions containing cyclosophoraoses were pooled, concentrated, and desalted by a Sephadex G-15 column (2 × 27 cm) under the conditions described above.

Preparation of anionic cyclosophoraoses. The desalted sample was then applied to a column (2 × 35 cm) of DEAE-cellulose to separate neutral and anionic cyclosophoraoses.

The column was first washed with distilled water containing 1 mM KCl, and a concentration gradient was applied from 1 mM to 100 mM KCl.

Electrospray ionization mass spectrometry. ESI-MS of the anionic cyclosophoraoses was carried out on a Micromass QuattroII (Altrincham, UK) double quadrupole mass spectrometer equipped with an ion spray source and connected to a syringe pump for sample injection. Electrospray spectra were recorded in the negative mode with the ionspray voltage set at 3.50 kV and the cone voltage at 30 V. Scanning was performed in the multi-channel analyzer mode from m/z 500 to m/z 4000. The samples were dissolved in water/acetonitrile (1 : 1, v/v) at a final concentration of about 0.5 mg/mL. The sample solution was introduced into the ESI source at a constant flow rate of 5 μL/min, 80 °C.

NMR spectroscopy of anionic cyclosophoraoses. NMR spectroscopic analyses were performed on a Bruker AMX spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C) at 25 °C. The purified anionic cyclosophoraoses were dissolved in deuterated water (D_2O, 99.96%). ^{31}P NMR spectra were recorded with a Bruker DRX-500 NMR spectrometer operating at a ^{31}P frequency of 202 MHz. A sample of 20 mg was dissolved in D_2O with an external standard solution of inorganic phosphoric acid. Measurements were performed in a 5 mm probe using 5 mm sample tubes at 25 °C.

Results and Discussion

NMR spectroscopic analysis. The presence of glycerol-1-phosphates in the anionic cyclosophoraoses was confirmed by NMR spectroscopic analysis. Each of the peaks at 66.8, 71.1, 62.5 ppm was assigned to C-1', C-2', and C-3' of *sn*-glycerol-1-phosphate residues, respectively. Each resonance of C-1 to C-6 of anionic cyclosophoraoses was also assigned at 102.4, 83.1, 75.8, 69.1, 76.7, and 61.0 ppm,

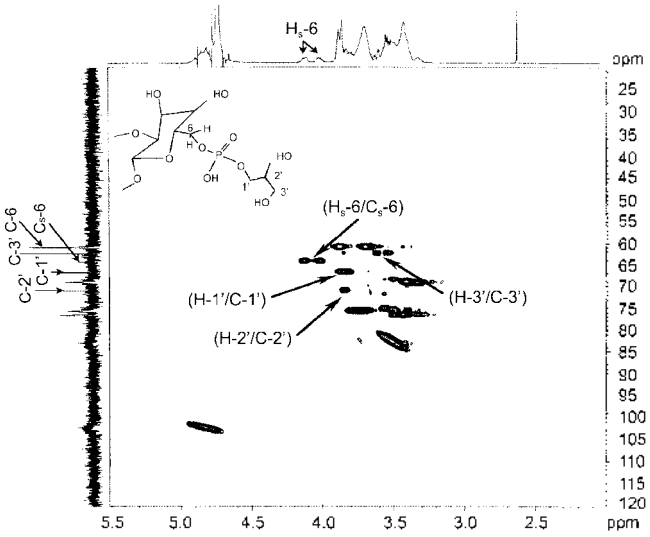

Figure 2. HSQC spectrum of anionic cyclosophoraoses. *sn*-glycerol-1-phosphate residues were directly linked to the C-6 of glucose residue through a phosphodiester linkage. (C_s-6 and H_s-6: C-6 and H-6 of the glucose residue substituted with glycerol-1-phosphate).

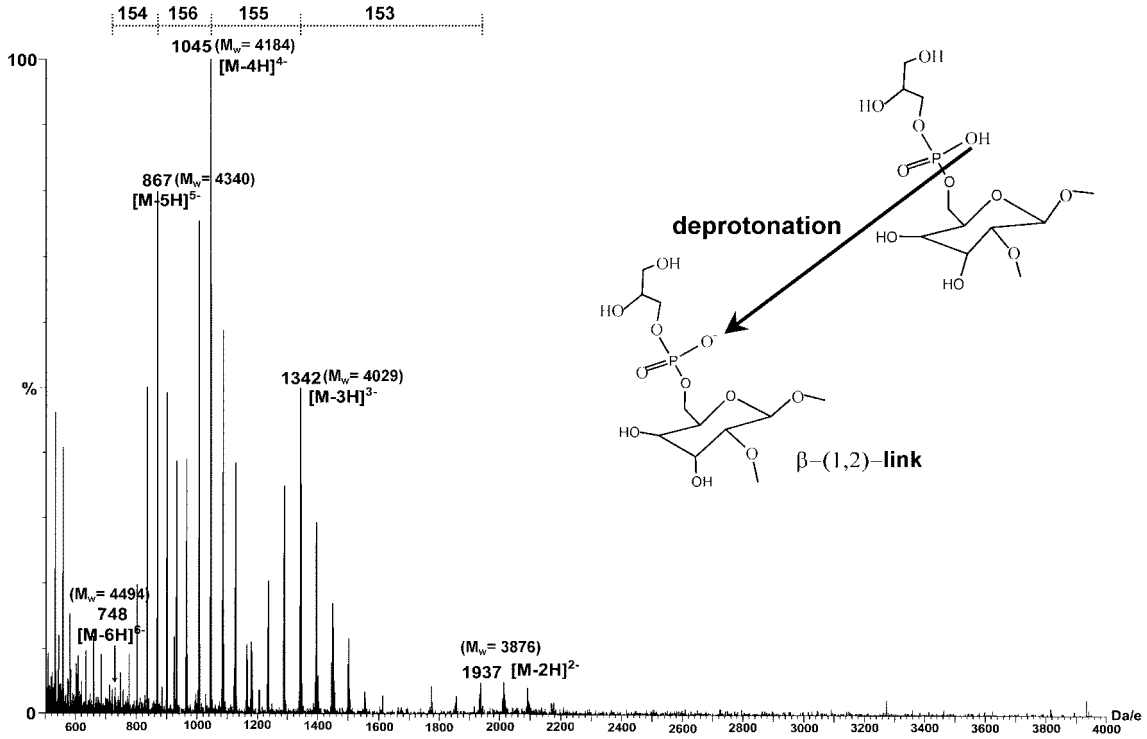

Figure 3. ESI mass spectrum of anionic cyclosophoraoses. The mass units at 748, 867, 1045, 1342 and 1937 correspond to the anionic cyclosophoraoses substituted with 2 to 6 glycerol-1-phosphate moieties in DP 22, respectively. Calculated molecular weights (M_w) were shown in parenthesis. Mass difference of 155 corresponds to the molecular weight of glycerol-1-phosphate. (Inner diagram: The deprotonation scheme of anionic cyclosophoraoses.)

Table 1. Expected and observed mass units of total anionic cyclosophoraoses isolated from *R. meliloti* 2011 (Expected: The calculated values, Observed: The values acquired from ESI mass spectrum)

			DP (Degree of Polymerization)										
			17	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
The number of substitution of phosphoglycerol	**2**	Expected	1531	1612	1693	1774	1855	1936	2017	2098	2179	2260	2341
		(Observed)	(1532)	(1613)	(1693)	(1775)	(1856)	(1937)	(2014)	(2091)	(2179)	***n.d***	***n.d***
	3	Expected	1072	1126	1180	1234	1288	1342	1396	1450	1504	1558	1612
		(Observed)	***n.d***	(1126)	(1180)	(1234)	(1288)	(1342)	(1396)	(1448)	(1502)	(1556)	(1613)
	4	Expected	843	883	924	964	1005	1045	1086	1126	1167	1207	1248
		(Observed)	***n.d***	(883)	(924)	(964)	(1005)	(1045)	(1086)	(1126)	(1165)	(1207)	(1248)
	5	Expected	705	737	770	802	834	867	899	932	964	996	1029
		(Observed)	***n.d***	***n.d***	(770)	(802)	(835)	(867)	(899)	(932)	(964)	***n.d***	(1028)
	6	Expected	613	640	667	694	721	748	775	802	829	856	883
		(Observed)	***n.d***	***n.d***	***n.d***	***n.d***	(721)	(748)	(775)	(802)	(829)	***n.d***	(857)

n.d: not detected.

respectively (data not shown). Phosphate substitution that occurs at C-6 of the glucose residues resulted in shift of the glucose C-4, C-5, and C-6 resonance. Particularly, the C-6 peak in substituted glucose residues shifted to 64.4 ppm because of the anionic substitution. The chemical shifts of C-4, C-5 resonances also moved a little to an upfield area. Figure 2 shows the heteronuclear single quantum coherence (HSQC) spectrum of isolated anionic cyclosophoraoses. Methylene groups bound to the C-6 of glucose residues substituted with *sn*-glycerol-1-phosphate and the three carbons of glycerol were assigned in Figure 2. It showed the C-6 of substituted glucose residues was directly bound to the phosphate of anionic cyclosophoraoses. Upfield Chemical shift of the C-1' of glycerol also indicated that the phosphate was linked to that position. On the while, the presence of phosphate groups of anionic cyclosophoraoses was confirmed by ^{31}P-NMR spectroscopy (data not shown). Thus, in the case of anionic cyclosophoraoses synthesized from *R. meliloti* 2011, only *sn*-glycerol-1-phosphate residues were directly linked to some glucose residues through phosphodiester linkages at the C-6 position.

ESI-Mass spectrometry. Figure 3 shows the total mass spectrum of the anionic cyclosophoraoses of *R. meliloti* 2011. Spectrum was acquired in the negative-ion mode by deprotonation, $[M-H]^-$. The pattern of this spectrum looked similar to that of isolated fraction from DEAE-cellulose chromatography.[7,21] The ESI-MS spectrum of anionic cyclosophoraoses contained -2 ($[M-2H]^{2-}$) to -6 charged ($[M-6H]^{6-}$) molecules with complex peak sets formed. The mass units at 748, 867, 1045, 1342 and 1937 correspond to the

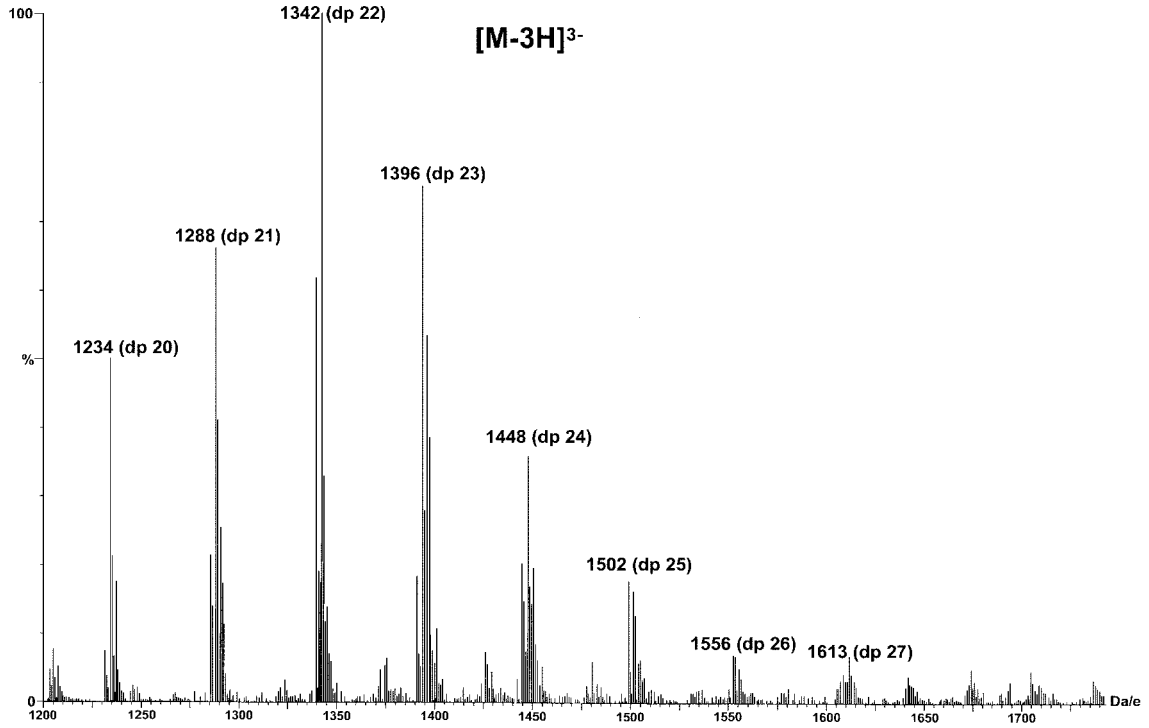

Figure 4. ESI mass spectrum of $[M-3H]^{3-}$ of anionic cyclosophoraoses.

anionic cyclosophoraoses of the degree of polymerization (DP) 22, respectively. The molecular weight (Mw) difference between the major peaks is about 155 corresponding to one phosphoglycerol moiety as a substituent. On the while, most major peaks of anionic cyclosophoraoses appeared around DP 22, which were also major in neutral ones in *R. meliloti* 2011.[22]

Each of expected or observed masses of the anionic cyclosophoraoses of Figure 3 was sorted out in Table 1. All the masses of observed peaks were excellently explained by the prediction based on the charged glycerol-1-phosphate substituents. The degree of substitution of anionic moieties was turned out to be dependent on the DP (degree of polymerization) of cyclosophoraoses (Table 1). The larger DP of cyclosophoraoses, the more degree of substitutions of glycerol-1-phosphate moieties was evidently observed. In the anionic cyclosophoraoses larger than DP 21, two to six glycerol-1-phosphate moieties were attached to the cyclosophoraoses. However, in the case of DP 17, only two glycerol-1-phosphate moieties were bound to the cyclosophoraoses. This observation clearly indicates that not all the neutral cyclosophoraoses are substrates for anionic ones. Depending on their sizes, the degree of anionic substitution was changed. It might give an answer why some of cyclosophoraoses are so large in degree of polymerization like DP 40. The larger cyclosophoraoses can possess the more charged moieties within the neutral ones so that microorganism can be more effectively adapted to external osmotic regulation. Figure 4 shows the ESI mass spectrum of $[M-3H]^{3-}$ anionic cyclosophoraoses substituted with three glycerol-1-phosphate molecules, which clearly indicated that each of the mass differences between adjacent major peaks was 162 corresponding to one glucose unit.

In the present study, we showed that anionic cyclosophoraoses contained the C-6 of substituted glucose residues, directly bound to the phosphate of *sn*-glycerol-1-phosphate and used first ESI-MS to find out the dependence of DAS (degree of anionic substitution) of cyclosophoraoses on their DPs based on the analysis of molecular weight distribution of whole anionic cyclosophoraoses isolated from the *R. meliloti* 2011. Further application for inclusion complexation with these anionic cyclosophoraoses is now under investigation.

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