Carbohydrate Research 343 (2008) 2422–2427

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Structural characterization of neutral and anionic glucans from *Mesorhizobium loti*

Yasuyuki Kawaharada [a], Hiromasa Kiyota [b], Shima Eda [a], Kiwamu Minamisawa [a], Hisayuki Mitsui [a,*]

[a] Graduate School of Life Sciences, Tohoku University, Katahira, Aoba-ku, Sendai 980-8577, Japan
[b] Graduate School of Agricultural Science, Tohoku University, Tsutsumidori-Amamiya-machi, Aoba-ku, Sendai 981-8555, Japan

ARTICLE INFO

Article history:
Received 28 May 2008
Received in revised form 10 July 2008
Accepted 11 July 2008
Available online 18 July 2008

Keywords:
Mesorhizobium loti
Cyclic glucan
β-(1→2)-Glycosidic linkage
Glycerophosphoryl substituent
Succinyl substituent

ABSTRACT

The periplasmic glucans of *Mesorhizobium loti* were isolated and separated into fractions according to their acidity. NMR spectroscopy confirmed their backbone structure to be a cyclic β-(1→2)-D-glucan as in the case of other rhizobia, and revealed no non-glycosidic substituents in the neutral fraction, and glycerophosphoryl and succinyl residues as major and minor substituents, respectively, in the anionic fractions. MALDI-TOF mass spectrometry showed that the anionic glucans contain one, two, or three such substituents per molecule according to their acidity, and, in contrast, that all the anionic subfractions have a similar size distribution to that of the neutral glucans, where molecules composed of 20–24 glucosyl residues are predominant. These results clarify the periplasmic glucan composition in terms of charge-to-mass ratios in *M. loti* cells.

1. Introduction

Gram-negative bacteria generally contain low-molecular-weight glucose polymers in the periplasm. Despite their structural diversity, such periplasmic glucans are considered in common to play an essential role in the envelope organization, and mutants defective in the biosynthesis show a pleiotropic phenotype including a defect in hypo-osmotic adaptation.[1] Moreover, in the case of pathogenic or symbiotic bacteria, periplasmic glucans are known to be crucial for the interaction with their eukaryotic hosts; as an example of their roles, periplasmic glucans were reported to suppress the host defense/immune mechanisms for plant symbiont *Bradyrhizobium japonicum*, mammalian pathogen *Brucella abortus*, and plant pathogen *Xanthomonas campestris* pv. *campestris*.[2–4] In the case of *Brucella*, it has been proposed that the function of the molecule is to extract cholesterol from eukaryotic membranes so as to disrupt cholesterol-rich lipid rafts present on phagosomal membranes.[5] As for the family *Rhizobiaceae*, which includes various pathogenic or symbiotic species, cyclic β-(1→2)-D-glucans have been determined to be their periplasmic glucans from the structural analyses with various bacteria such as *Agrobacterium*, *Brucella*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* species.[6–13] The molecules consist of β-(1→2)-linked D-glucosyl residues with the degrees of polymerization (DPs) ranging between 17 and 28, and some residues are modified by non-glycosidic substituents; the substituents are phosphoglycerol in *A. tumefaciens*,[14] succinic acid in *B. abortus*,[15] both in *S. meliloti*,[16] and both methylmalonic acid and succinic acid in *R. radiobacter*,[17] but no substituents in *M. huakuii*.[13] Such substituents confer negative charge on the glucans, which would lead to the change in cell-envelope characteristics. Whereas the anionic glucans are divided into more than one fraction according to the acidity within one organism, the charge-to-mass ratio has not been accurately characterized for each of the fractions.

Rhizobia are distinguished by the symbiotic nitrogen fixation with leguminous plants. *S. meliloti* is accepted as a model species for molecular genetics that establishes symbiosis with plants forming indeterminate-type nodules (e.g., alfalfa), the one of the major nodule types. The mutants for cyclic β-(1→2)-D-glucan synthesis were shown to be defective in the infection of host plants.[18] *Mesorhizobium loti* is another model species that establishes symbiosis with plants forming determinate-type nodules (e.g., *Lotus japonicus*), the other of the major nodule types. Its periplasmic glucans, however, have not been structurally characterized, whereas some *M. loti* studies dealt with the glucans through the chromatographic analyses.[19,20] We reported a novel *M. loti* mutant that shows defective infection of *L. japonicus*. It was suggested that the *cep* gene product, designated in that work, acts on a successful symbiosis by affecting the content of periplasmic glucans through the genetic and chromatographic analyses.[21] Thus, we attempted in this work to determine the structure of the glucans, with special regard to the charge-to-mass ratios of the molecules.

* Corresponding author. Tel.: +81 22 217 5685; fax: +81 22 217 5684.
E-mail address: hmitsui@ige.tohoku.ac.jp (H. Mitsui).

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doi:10.1016/j.carres.2008.07.007

2. Results and discussion

2.1. *M. loti* contains cyclic β-(1→2)-D-glucans partially substituted with phosphoglycerol and succinic acid

M. loti cells were extracted with 70% ethanol, and the extract was subjected to gel-filtration chromatography. The glucan fractions, which occurred as a major peak having a K_{av} of 0.45–0.7 as previously shown,[21] were pooled and then analyzed by anion-exchange chromatography. The chromatogram displayed five peaks, indicating one neutral (N) and four anionic (A1 to A4) subfractions (Fig. 1). This elution profile is similar to that reported previously except for an additional peak (A4).[21] Each of these subfractions was subjected to nuclear magnetic resonance (NMR) spectroscopy.

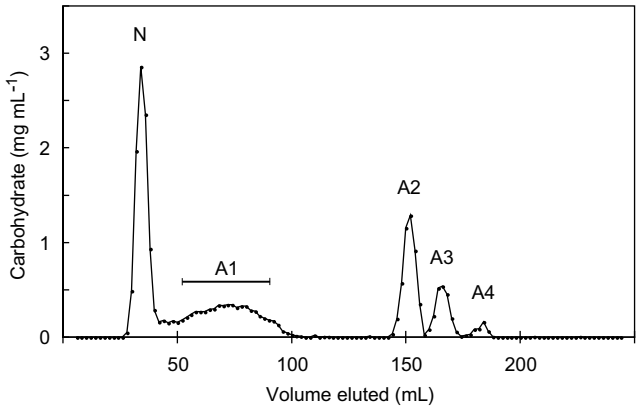


Figure 1. Separation of total glucans from *Mesorhizobium loti* by anion-exchange chromatography. A glucan fraction from the gel filtration (see text) was applied to a Q-Sepharose column (2.6 × 10 cm), which was eluted first with 85-mL buffer with no NaCl and then with a 150-mL linear gradient beginning with 0 mM and ending with 400 mM NaCl in the same buffer. Fractions (2 mL) were collected and assayed for total carbohydrate. The neutral peak is marked N, and anionic peaks are marked A1, A2, A3, and A4, respectively. The bar indicates fractions collected for A1. The carbohydrate amounts included in the N, A1, A2, A3, and A4 fractions are 17.8 mg, 11.1 mg, 8.9 mg, 3.9 mg, and 0.8 mg, respectively.

The N fraction showed a ^{1}H NMR spectrum close to those reported previously for cyclic β-(1→2)-D-glucans, in which the resonance assignments were established to protons H-1 to H-6 of the glucosyl residue (Fig. 2a).[9] The spectra of the anionic subfractions A1 to A3 were slightly different from that of N (Fig. 2b for A2; data not shown for A1 and A3); a similar spectrum was previously described for anionic cyclic β-(1→2)-D-glucans containing glycerophosphoryl and succinyl substituents.[22] Evidence about the presence of phosphorus (atom) was provided by ^{31}P NMR spectroscopy, and resonances were as singlets at 1.5 ppm for A1 to A3, supporting the presence of phosphoglycerol as an anionic substituent. The ^{1}H NMR spectra also indicate that each of the above subfractions is almost exclusively composed of glucans. In contrast, the A4 subfraction showed a quite different complex ^{1}H NMR spectrum from those of the others (data not shown), indicating that A4 is a mixture of carbohydrates of different sugar compositions and that glucans represent only a small percentage, if any, of the mixture; it has not been further characterized.

To confirm the structural characteristics suggested above, we performed ^{13}C NMR analysis for the A2 subfraction. The resonance assignment indicated in Table 1 was done by ^{1}H–^{1}H COSY, HSQC, and HMBC experiments (data not shown). The spectrum showed resonances attributable to respective carbons of a β-(1→2)-linked D-glucosyl residue as reported previously for unsubstituted molecules; several signals were observed close together at the chemical

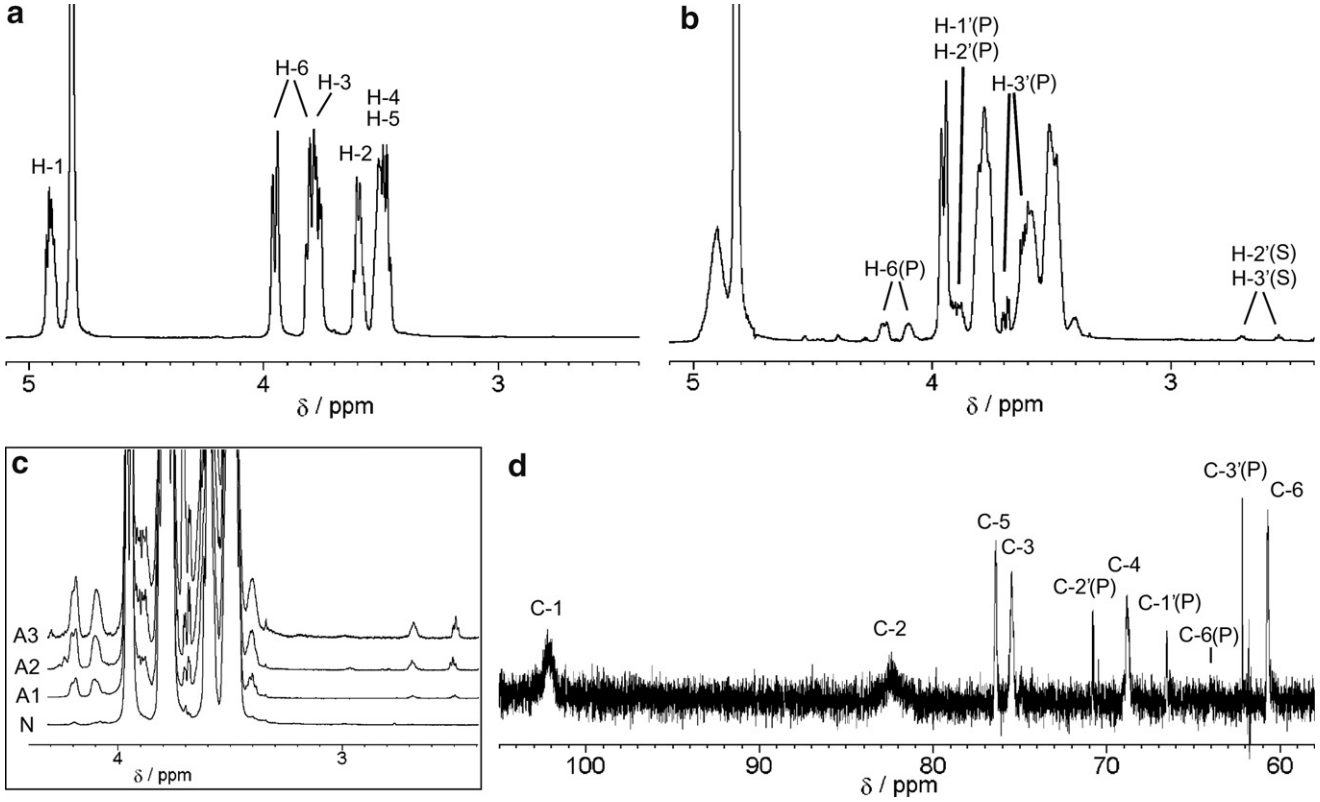
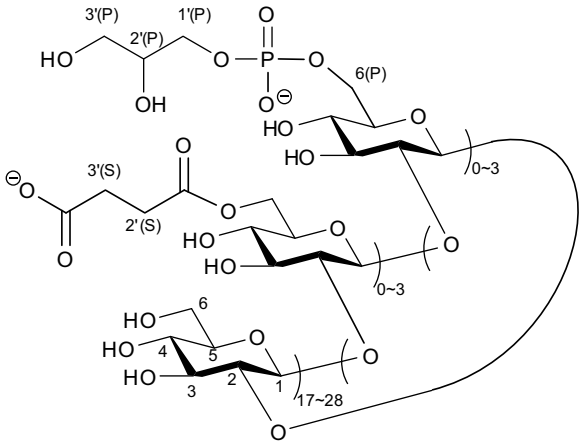


Figure 2. NMR spectroscopy of cyclic glucans from *Mesorhizobium loti*. (a) ^{1}H NMR spectrum of the N fraction. (b) ^{1}H NMR spectrum of the A2 fraction. Chemical shifts are expressed relative to internal DSS. The resonance at 4.82 ppm is HOD. (c) Peak areas are compared among the N, A1, A2, and A3 fractions as normalized to the H-6 signal at 3.95 ppm. (d) ^{13}C NMR spectrum of the A2 fraction. The used glucan fractions are shown in Figure 1. The resonance assignment indicated was done by ^{1}H–^{1}H COSY, HSQC, and HMBC.

Table 1
^{1}H, ^{13}C, and ^{31}P NMR spectral data of the cyclic glucans in D_2O



Position	δ_C	δ_H (mult.,[a] in *J* Hz)	δ_P
C-1	102.3–102.5	4.90 (s)	
C-2	82.1–82.9	3.56 (br)	
C-3	75.4–75.6	3.78 (br)	
C-4	68.7–68.9	3.46 (br)	
C-5	76.4–76.5	3.49 (br)	
C-6	60.7–60.8	3.75 (br)	
		3.95 (d, 12.2)	
C-6(P)	64.0	4.10 (br)	
		4.19 (br d, 10.8)	
P			1.5 (s)
C-1′(P)	66.5	3.86 (m)	
		3.93 (m)	
C-2′(P)	70.8	3.89 (m)	
C-3′(P)	62.2	3.62 (dd, 11.7, 5.7)	
		3.69 (dd, 11.7, 8.2)	
C-1′(S)	178.5[b]		
C-4′(S)	175.4[b]		
C-2′(S)		2.50 (t, 6.8)	
C-3′(S)		2.68 (m)	

[a] Multiplicity: s, singlet; d, doublet; t, triplet; br, broad (singlet).
[b] The chemical shift values were elucidated from the HMBC spectrum.

shifts where C-1, C-2, and C-4 are expected to be observed; the multiple signals are due to the variation in ring size of molecules (see below) (Fig. 2d).[6] The absence of signals at ~74 ppm and at 92–96 ppm indicates that non-reducing-terminal glucosyl groups and reducing glucose residues are not present, respectively, in the molecules, confirming the cyclic nature of the molecule.[6]

Resonances at 66.5, 70.8, and 62.2 ppm are assigned to C-1′, C-2′, and C-3′, respectively, of the glycerophosphoryl residue as described previously.[8] A weak signal at 64.0 ppm is assigned to C-6 connecting to phosphoglycerol, which is consistent with the previous report indicating downfield shift of the C-6 resonance due to such substitution.[8] In ^{1}H NMR spectra of the anionic glucans, two clear signals at 4.10 and 4.19 ppm are assigned to H-6 geminal protons linked to C-6 with the glycerophosphoryl substituent as previously described (Fig. 2b).[23] The intensity of both signals increased serially from A1 to A3; the ratio of peak areas at 4.19 ppm is 1:2.1:3.1 for A1:A2:A3 as normalized to those of H-6 at 3.95 ppm (Fig. 2c).

In addition, a pair of small triplets was detected at 2.50 and 2.68 ppm in the ^{1}H NMR spectra of A1, A2, and A3 but not in that of N; the intensity of both signals increased serially from A1 to A3 (Fig. 2b and c). These signals displayed a mutual correlation but not with the other protons in the ^{1}H–^{1}H COSY spectrum (data not shown), suggesting that they are assigned to H-2′ and H-3′ methylene protons of succinyl residues as previously described.[15] Moreover, they showed correlations with carbonyl carbons, which would be attributable to C-1′ and C-4′ of succinic acid, in the HMBC spectrum (data not shown). These results suggest the presence in small amounts of succinyl substituents, although virtually no methylene carbon signals could be detected in the ^{13}C NMR spectrum. The ratios of a peak area at 2.50 ppm to that at 4.19 ppm are about 1:9 in A1 and 1:5 in A2 and A3, which are putative molar ratios of succinyl residues to glycerophosphoryl residues present in glucan subfractions. Not so variable ratios among subfractions suggest that both of the moieties are transferred to glucan backbones randomly regardless of their prior substitution, presuming that N, A1, and A2 are the biosynthetic precursors of A1, A2, and A3, respectively, as previously described.[24]

In conclusion, *M. loti* cells contain cyclic β-(1→2)-D-glucans like the other rhizobia examined so far, and these are divided into neutral glucans with no substituents and anionic glucans with phosphoglycerol as a major substituent and, most likely, succinic acid as a minor substituent; the amount of substituents increases per glucosyl residue as the acidity of molecules increases. Here we noticed the *M. loti* gene *mlr8375*, which is annotated as *opgC* encoding a succinyl transferase to periplasmic glucans,[25] in the whole-genome sequence of *M. loti* (RhizoBase, http://bacteria.kazusa.or.jp/rhizobase/). This would be another basis for the presence of succinyl substituents.

2.2. Fractions of cyclic glucans have different numbers of substitutents per molecule but similar ring-size distributions

We analyzed *M. loti* cyclic β-(1→2)-D-glucans with each subfraction separately by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry in a positive-ion mode. The spectrum from the N fraction showed a series of 12 sodium-cationized molecular ions $[M+Na]^+$, each of which occurred at an interval of about 162 mass unit (Fig. 3a). These ion species have the same masses as expected for cyclic glucans composed of 17–28 glucosyl residues with no substituents (Table 2). Among them, molecules with DPs ranging from 20 to 24 seem to be predominant. This is similar to the case of *M. huakuii*, where the dominant DP is 22 in the range between 17 and 28.[13]

The spectrum from the A1 subfraction revealed a series of molecular ions paralleling that from the N fraction, but each of the ion species $[M-H+2Na]^+$ is larger by about 176 mass unit than the corresponding species from N (Fig. 3b). The difference corresponds to a mass expected for one sodiophosphoglycerol moiety (Table 2). In addition, a weak signal at *m*/*z* 3687.5 would correspond to an ion species $[M+Na]^+$ having a mass expected for the sum of 22 glucosyl residues and one succinyl residue.

The spectrum of the A2 subfraction seems to be a complex of two series of sodium-cationized molecular ions, each of which parallels that from the N fraction (Fig. 3c). The major series, which consists of ion species $[M-2H+3Na]^+$ at *m*/*z* 3454.6, 3615.6, 3777.6, 3939.6, 4101.5, 4263.4, 4425.3, and 4587.3, is up-shifted by about 352 mass unit as compared with that from N; the difference corresponds to a mass expected for two sodiophosphoglycerol moieties (Table 2). In contrast, signals at *m*/*z* 3539.8, 3863.7, and 4025.6 would correspond to ion species $[M-H+2Na]^+$ having masses expected for the sum of 20, 22, and 23 glucosyl residues, respectively, one sodiophosphoglycerol moiety and one succinyl moiety (Table 2). The A3 subfraction showed a more complex spectrum (Fig. 3d). Compared with that from N, the major series of ion species $[M-3H+4Na]^+$ (*m*/*z* 3624.0, 3791.3, 3953.3, 4115.3, 4277.2, 4439.2, 4601.0, and 4764.0) is up-shifted by about 528 mass unit, which corresponds to a mass expected for three sodiophosphoglycerol moieties (Table 2). In contrast, other signals would correspond to ion species $[M-2H+3Na]^+$ having masses expected for glucan molecules containing each of two sodiophosphoglycerol moieties and one succinyl moiety, ion species $[M-H+2Na]^+$ having masses

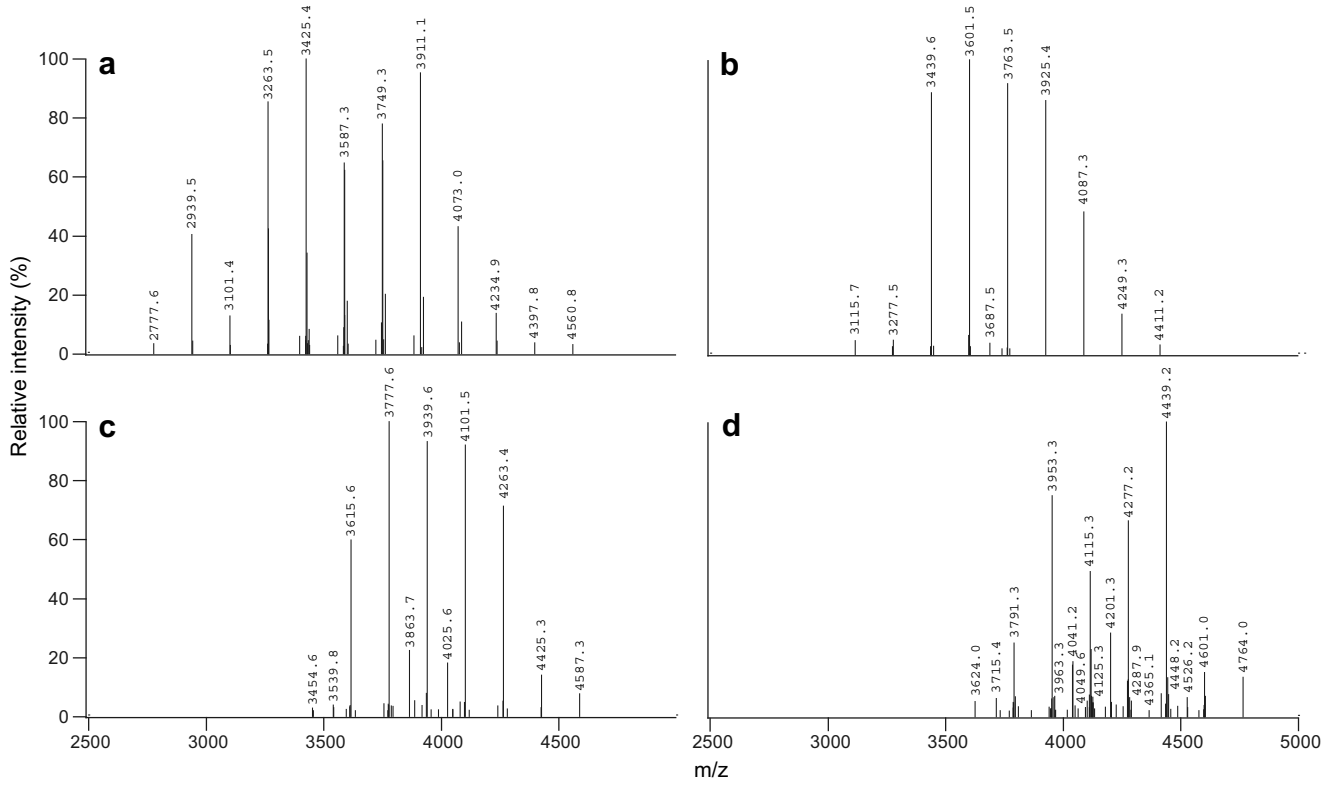


Figure 3. Positive-ion mass spectra of cyclic glucans from *Mesorhizobium loti*. Numbers over peaks indicate measured masses of ion species that are estimated to be sodium-cationized glucans. Glycerophosphoryl and succinyl residues within glucan molecules are presumed to take the forms of sodium salt and free acid, respectively. (a) N fraction. (b) A1 fraction. (c) A2 fraction. (d) A3 fraction. The used glucan fractions are shown in Figure 1.

Table 2
Numbers of residues per glucan molecule predicted from masses of sodium-cationized ion species detected in mass spectra

Fraction	Measured masses	Numbers per molecule			Fraction	Measured masses	Numbers per molecule		
		Glucose	Phosphoglycerol	Succinic acid			Glucose	Phosphoglycerol	Succinic acid
N	2777.6	17	0	0	A1	3115.7	18	1	0
	2939.5	18	0	0		3277.5	19	1	0
	3101.4	19	0	0		3439.6	20	1	0
	3263.5	20	0	0		3601.5	21	1	0
	3425.4	21	0	0		3687.5	22	0	1
	3587.3	22	0	0		3763.5	22	1	0
	3749.3	23	0	0		3925.4	23	1	0
	3911.1	24	0	0		4087.3	24	1	0
	4073.0	25	0	0		4249.3	25	1	0
	4234.9	26	0	0		4411.2	26	1	0
	4397.8	27	0	0					
	4560.8	28	0	0					
A2	3454.6	19	2	0	A3	3624.0	19	3	0
	3539.8	20	1	1		3715.4	20	2	1
	3615.6	20	2	0		3791.3	20	3	0
	3777.6	21	2	0		3953.3	21	3	0
	3863.7	22	1	1		3963.3	22	1	2
	3939.6	22	2	0		4041.2	22	2	1
	4025.6	23	1	1		4049.6	23	0	3
	4101.5	23	2	0		4115.3	22	3	0
	4263.4	24	2	0		4125.3	23	1	2
	4425.3	25	2	0		4201.3	23	2	1
	4587.3	26	2	0		4277.2	23	3	0
						4287.9	24	1	2
						4365.1	24	2	1
						4439.2	24	3	0
						4448.2	25	1	2
						4526.2	25	2	1
						4601.0	25	3	0
						4764.0	26	3	0

expected for glucan molecules containing each of one sodiophosphoglycerol moiety and two succinyl moieties, or ion species $[M+Na]^+$ having masses expected for glucan molecules containing three succinyl moieties (Table 2).

According to the spectra from A1 to A3 with the above interpretation, DPs ranging between 20 and 24 are predominant for the anionic glucans as for the neutral glucans.

The mass analyses indicate that the anionic subfractions A1, A2, and A3 of the *M. loti* cyclic β-(1→2)-D-glucans contain one, two, and three non-glycosidic substituents, respectively, per glucan molecule. The results also support the presence of glycerophosphoryl and succinyl residues as major and minor substituents, respectively, in the anionic glucans. Moreover, a similar ring-size distribution between neutral and anionic fractions demonstrated convincingly that there is no preference for a particular DP for transfer of non-glycosidic moieties as previously suggested.[15]

2.3. Conclusions

This work confirmed the presence of cyclic β-(1→2)-D-glucans, composed of neutral and anionic fractions, in *M. loti* cells and revealed the numbers of anionic substituents as well as glucosyl residues per glucan molecule for each fraction; these results clarified the cyclic glucan composition in terms of charge-to-mass ratios within one organism for the first time.

The reason for the negative charge of the periplasmic glucan remains to be investigated. Among rhizobia, broad-host-range *Rhizobium* sp. GRH2, *R. leguminosarum* bv. *trifolii* TA-1, and *M. huakuii* IFO15243 produce cyclic glucans with no anionic substituents.[10,13,26] It therefore appears that anionic substituents are not absolutely required for symbioses between rhizobia and legumes. Anionic substituents of the periplasmic glucans have been suggested to strongly influence the Donnan potential across the outer membrane due to a net negative charge; they would therefore make an important contribution to the maintenance of osmolarity of the periplasm against the low osmolarity of the growth medium.[27] However, it is not likely that osmoprotection is the sole function of anionic substituents, because of the cases where periplasmic glucans are not substituted; the negative charge of periplasmic glucans would have a great influence on cell-envelope characteristics. We have to pay attention to the relationship between the cellular abundance and negative charge of periplasmic glucans to keep their functional effectiveness.

3. Experimental

3.1. Bacterial strain and culture condition

M. loti ML001, a streptomycin-resistant derivative of *M. loti* wild-type strain MAFF303099,[21] was grown at 30 °C with shaking in glutamic acid–D-mannitol–salts medium with a modification of the D-mannitol concentration to 1.0 g/L.[7]

3.2. Isolation of neutral and anionic glucans

M. loti cell pellets harvested from 8-L culture grown to an optical density at 660 nm of 0.7 were extracted with 240 mL of 70% (v/v) ethanol at 70 °C for 1 h. The extract was subjected to gel filtration on a HiPrep 16/60 Sephacryl S-100 HR column (1.6 × 60 cm; GE Healthcare). Fractions containing glucans were pooled, concentrated, and desalted. The sample (5 mL) was applied to a HiLoad 26/10 Q-Sepharose HR column (2.6 × 10 cm; GE Healthcare), which was first eluted with 85 mL of 10 mm Tris·HCl (pH 7.4) containing 7% (v/v) 1-propanol, and then with 150 mL of a linear gradient beginning with 0 mM and ending with 400 mM NaCl in the same buffer. Fractions (2 mL) were assayed for total carbohydrate content as glucose equivalents by the anthrone/sulfuric acid method.[28]

3.3. NMR spectroscopy

^1H, ^{13}C, and ^{31}P NMR spectra were recorded with Varian Inova 600 (600 MHz for ^1H and 151 MHz for ^{13}C) and Inova 500 (202 MHz for ^{31}P) spectrometers at 20 °C in D_2O with sodium 3-(trimethylsilyl)propanesulfonate (DSS, δ_H 0 ppm) as an internal standard. The ^{13}C and ^{31}P chemical shift values were not corrected.

3.4. Mass spectrometry

MALDI-TOF mass spectra were recorded with Voyager-DE STR Biospectrometry workstation (Applied Biosystems) in a positive-ion mode using 2,5-dihydroxybenzoic acid as the matrix. ProteoMass/insulin (bovine) (5730.6087 $[M+H^+]$; Sigma) and Peptide Calibration Standard (ACTH clip 18-39, 2465.1983 $[M+H^+]$; somatostatin 28, 3147.4710 $[M+H^+]$; Bruker Daltonics) were used as external standards for calibration.

Acknowledgments

We thank Mrs. Teiko Yamada (Tohoku University) for technical assistance on NMR spectroscopy. This work was supported in part by Grant-in-Aid for Scientific Research (No. 19580077) from Japan Society for the Promotion of Science, and by Grant-in-Aid for Scientific Research on Priority Areas 'Comparative Genomics' from the Ministry of Education, Culture, Sports, Science and Technology.

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