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Characterization of cyclic β-glucans of *Bradyrhizobium* by MALDI-TOF mass spectrometry

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ABSTRACT

Periplasmic, cyclic β-glucans isolated from *Bradyrhizobium elkanii*, *Bradyrhizobium liaoningense*, and *Bradyrhizobium yuanmingense* strains have been investigated by means of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS), 1D and 2D nuclear magnetic resonance (NMR), as well as standard chemical methods. These compounds are built of 10–13 D-glucose residues. The main fractions contain molecules assembled of 12 hexose units (M_w = 1945.363 Da). Glucose monomers are linked by β-(1→3) or β-(1→6) glycosidic bonds. The ratio of β-(1→3) to β-(1→6) linked glucose is approximately 1:2. Moreover, methylation analysis demonstrated the presence of terminal, non-reducing, as well as branched (i.e., 3- and 6-substituted) glucoses. Thus, the basic structure of the investigated compounds is similar to that of periplasmic oligosaccharides from *Bradyrhizobium japonicum* and *Azorhizobium caulinodans* strains. The analyzed cyclic β-glucans are substituted by phosphocholine (PC) (one or two residues per ring) and highly decorated with acetate and succinate. The substituents are arranged diversely in the population of cyclic β-glucan molecules. The concentrations of cyclic β-glucans in *Bradyrhizobium* periplasmic space are osmotically regulated and increase in response to a decrease of medium osmolarity.

1. Introduction

Symbiotic interactions between rhizobia and leguminous plants have been intensively studied for many years. These interactions lead to infection of plant tissues and formation of nodules (on roots or on stems) inside which rhizobia, transformed to bacteroids, can fix atmospheric nitrogen. Bacterial cell surface carbohydrates, that is, extracellular polysaccharides (EPS), capsular polysaccharides (CPS), lipopolysaccharides (LPS), and periplasmic β-glucans, are crucial to the process of establishing an effective symbiosis.[1] Miller et al.[2] as well as Tully et al.[3] have also shown that, in free-living conditions, cyclic β-glucans assist rhizobium strains in adapting to a hypoosmotic environment.

All species of the taxon Rhizobiales examined so far are capable of synthesizing cell-associated β-glucans. They are localized within the periplasmic compartment and may have a neutral or an anionic character.[1] β-glucans produced by *Rhizobium* species and *Agrobacterium tumefaciens* have been studied the most thoroughly. These oligosaccharides are cyclic and contain from 17 to 25 (in some cases as many as 40) β-D-glucose residues connected by β-(1→2) linkages. Cyclic β-glucans can be substituted with phosphoglycerol, succinate, methylmalonate, or acetate. In *Sinorhizobium meliloti* 1021, approximately 90% of the total periplasmic glucans have been found to be substituted with anionic moieties.[4] The predominant substituent is *sn*-1-phosphoglycerol linked to C-6 of glucose. Among non-sugar residues, phosphoglycerol, phosphoethanolamine, and phosphocholine derive from periplasmic membrane phospholipids, while acetyl, succinyl, and methylmalonyl originate from metabolic intermediates in the cytosol.[1,5]

Slow-growing rhizobia synthesize β-(1→3);(1→6)-glucans decorated with zwitterionic phosphocholine, which is attached to carbon C-6 of β-(1→3) linked glucose.[6] The size and size distribution of bradyrhizobial periplasmic β-glucans seems to be under a strict control. These glucans possess a ring composed of 11–13 glucosyl residues and are branched in structure, that is, they possess one terminal β-glucose.[1,6–8]

There is some evidence that the health of rhizobia depends on the proper structure of cyclic β-glucans. For example, *Bradyrhizobium japonicum* which was not capable of synthesizing β-(1→3);(1→6) glucans showed defective motility, was sensitive to hypoosmotic medium, and formed ineffective nodules.[1,8,9] Three genes coding the biosynthesis pathway of cyclic β-glucans have been identified in *B. japonicum* (*ndvB*, *ndvC* and *ndvD*), but only two of them (*ndvB* and *ndvC*) are required for the synthesis of cyclic β-(1→3);(1→6)-D-glucan and its successful symbiotic interaction with soybean (*Glycine max*). The product of the third gene influences the behavior of the bacteria and is not engaged in the synthesis and decoration of cyclic β-glucans. Several genes that encode

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enzymes necessary for the modification of β-glucan with non-sugar substituents have been identified so far.[1,10] Some of them catalyze identical reactions (for example, succinylation) despite not sharing any significant structural similarities.[10] Up till now, it has been generally accepted that bradyrhizobial cyclic β-glucans are substituted exclusively with the phosphocholine moiety. Here, we report results of structural analyses of β-glucans isolated from different species of the genus *Bradyrhizobium* which show that not only β-(1→2)-glucans but also β-(1→3);(1→6)-D-glucans may be highly substituted with succinate, acetate, and phosphocholine residues.

2. Results and discussion

The oligosaccharide materials extracted from the examined bacteria by means of the Bligh and Dyer procedure were separated on Sephadex G-50. Collected carbohydrate-containing fractions eluted closer to the permeation volume than a preparation of neutral cyclic β-glucans from *Mesorhizobium huakuii*[11] and had almost the same elution volume as β-glucans from *Azorhizobium caulinodans*.[12] These chromatographic properties indicated that the studied oligosaccharides were evidently smaller than those found in fast growing rhizobia and mesorhizobia and were comparable to those from *A. caulinodans*. Moreover, the Sephadex G-50-purified materials migrated similarly to the *Azorhizobium* β-glucan on TLC silica gel plates (data not shown). The obtained results indicated that the oligosaccharides extracted from *Bradyrhizobium elkanii* USDA 76, *Bradyrhizobium liaoningense* USDA 3622, and *Bradyrhizobium yuanmingense* CCBAU 10071^T were very similar to each other and also resembled the azorhizobial β-glucan, which, in turn, was almost the same as that isolated from *B. japonicum* USDA 110.[6,7]

In accordance with a suggestion by Miller et al.[7] in order to prevent losses of parts of cell-associated glucan fractions, which could be overlooked in the process of chromatographic elution because of the slight amounts of those fractions, we omitted fractionation on DEAE-cellulose. Salts and other small size molecules (impurities) were removed during dialysis using 1200 Da cut-off dialyzing tubes. The purified and desalted oligosaccharides, containing exclusively D-glucose, as confirmed by GC–MS analysis, were methylated, hydrolyzed, and analyzed by GC–MS in the form of permethylated alditol acetates. The results of these analyses are listed in Table 1. It should be noted that only permethylated glucose as well as methylated alditol acetates of glucose (acetyl groups at C-3 and/or C-6) were found, indicating that the obtained β-glucan preparations were typical of *Bradyrhizobium* and were not contaminated with other oligo- or exopolysaccharides. For comparison, we also present published data from methylation analyses of *B. japonicum* USDA 110 β-glucans[3,6,8] (Table 1). In general, in all the examined strains, the amounts of D-glucose substituted at position C-6 were twice as large as the amounts of glucose substituted at position C-3 (Table 1). Moreover, in all three *Bradyrhizobium* preparations, we observed that the amounts of doubly substituted glucose were always higher than the amounts of terminal glucose. This fact suggests that part of the glucose was substituted at position C-6 with extra residues which were not cleaved by the strong alkaline conditions of methylation and were removed later by a final acid hydrolysis in 2 M trifluoroacetic acid. The only substituent that could be taken into consideration is phosphocholine. The Hakomori[13] procedure of methylation partially removes (or destroys) phosphodiesters,[14] which is why their presence in β-glucan can influence the ratios of methylation products. The ^{1}H, ^{31}P HMQC spectrum of the *B. elkanii* β-glucan showed that at least two protons at 4.317 and 4.130 ppm are coupled with phosphorus at 0.88 ppm (data not shown). These protons are directly linked to carbons at 60.62 and 68.20 ppm, respectively. The above-mentioned data resemble those presented by Rolin et al.[6] and unequivocally indicate the presence of phosphocholine connected at position C-6 of glucose in the *B. elkanii* β-glucan. They also well explain the excess of disubstituted glucose units over terminal units in methylated cyclic glucan preparations.

A preliminary analysis of 1D ^{1}H NMR spectra of all three glucan samples revealed resonances characteristic of succinyl (δ_H 2.69 and 2.50 ppm for HOOC–C*H*$_2$–C*H*$_2$–COO–, respectively), acetyl (δ_H 2.10 ppm for C*H*$_3$–COO–), and phosphocholine (δ_H 3.20 ppm for –N$^+$(C*H*$_3$)$_3$) residue protons. Some differences observed among the ^{1}H NMR spectra were restricted to the intensities and/or the presence of resonances from non-sugar substituents. The neutral β-glucans of *B. elkanii* USDA 76 were analyzed in detail using 2D ^{1}H NMR spectra. As anion-exchange chromatography on a DEAE column[4,7] is ineffective for separation of neutral and phosphocholine-decorated β-glucans, the neutral cyclic glucan fraction (approx. 11 mg) was obtained by treating the β-glucan preparation with 12.5% ammonia and next with 48% HF (see Section 3). The ^{13}C and ^{1}H NMR resonances were assigned using 2D NMR data from ^{1}H, ^{1}H COSY, TOCSY, and ^{1}H, ^{13}C HSQC experiments. All chemical shifts are listed in Table 2. The anomeric configurations of glucose residues were found to be exclusively β as all $J_{H1,H2}$ coupling constants were around 8 Hz. We observed a considerable multiplicity of resonances assigned to carbons C-3 and C-6. A similar observation has been reported in detail by Rolin et al.[6] for a native β-glucan preparation from *B. japonicum* USDA 110. The 2D NMR spectra obtained for the *B. elkanii* USDA 76 neutral β-glucan were almost identical to those published for *B. japonicum* USDA 110[6] and were, therefore, not included in this work. The similarities arose from the fact that in both the neutral oligosaccharide fraction obtained from *B. elkanii* USDA 76 glucan and the substituted β-glucans from *B. japonicum* USDA 110 most of the sugar components did not bear any residues.[6] Obviously, our spectra, recorded for dephosphorylated and O-deacylated oligosaccharides, were deprived of non-sugar residue signals.

The ROESY spectrum of *B. elkanii* neutral β-glucan (data not shown) revealed two cross signals at 5.110/4.192 ppm and 5.110/3.882 ppm which pointed to position C-6 of C-3 substituted glucose as the site of attachment of the terminal glucose. Thus, since

Table 1
Percentage contents of permethylated alditol acetates obtained from β-glucans isolated from *Bradyrhizobium* strains

No.	Strain	t-Glc(1→	→3)Glc(1→	→6)Glc(1→	→3,6)Glc(1→
1.	*B. elkanii* USDA 76	8.5	27.6	53.9	10.0
2.	*B. liaoningense* USDA 3622	7.2	22.9	56.2	13.8
3.	*B. yuanmingense* CCBAU 10071	6.5	21.8	58.4	13.3
4.	*B. japonicum* USDA 110[a]	7.0	9.0	68.0	18.0
5.	*B. japonicum* USDA 110[b]	5.0	10.0	71.0	16.0
6.	*B. japonicum* USDA 110[c]	8.0	38.0	46.0	8.0

[a] Data taken from Ref. 3.
[b] Data taken from Ref. 8.
[c] Data taken from Ref. 6, t-Glc—terminal glucose.