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Note

The extracellular glucans of *Rhizobium japonicum* strain 3I1b71a

WILLIAM F. DUDMAN
Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, P.O. Box 1600, Canberra City, A.C.T. 2601 (Australia)
AND ALAN J. JONES
The National NMR Centre, Australian National University, Canberra, A.C.T. 2600 (Australia)
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The extracellular polysaccharides of three strains of *Rhizobium japonicum* (3I1b71a, CC708, and CB1795) have been shown to be mixtures of large proportions of acidic polysaccharides and small proportions of glucans[1]. The acidic polysaccharides have been characterized as rhamno-4-*O*-methylglucuronans, but the glucans were not examined further. The present note describes the isolation and examination of the glucan fraction present in the extracellular polysaccharides of *R. japonicum* strain 3I1b71a. This was found to be itself a mixture of two branched glucans, both composed of β-D-(1→3)-, β-D-(1→6)- and β-D-(1→3)(1→6)-linked residues and non-reducing terminal groups, but in different proportions in the two glucans.

The isolation of a sufficient quantity of the glucan fraction was a problem. The earlier investigation showed that the rhamno-4-*O*-methylglucuronan could be separated from the glucans by column chromatography, but that the recovery of the latter from the Sephadex columns was very low[1]. In the present investigation, the rhamno-4-*O*-methylglucuronan was converted into the barium salt and precipitated from the culture supernatant solution by addition of acetone to 75% by volume; this action co-precipitated some of the glucan, which was then separated by column chromatography and recovered in low yield ("C-" or "column" glucan, 70 mg). Some glucan also remained in the acetone-rich supernatant solution and was isolated separately as "S-" or "supernatant" glucan (150 mg).

Both glucans contained only glucose; susceptibility to oxidation by D-glucose oxidase confirmed it to be D-glucose. The elution pattern of C-glucan during its isolation on Sephadex G-200 columns indicated a single homogeneous component having an elution volume corresponding to a molecular weight of ~4500. Its homogeneity and molecular weight were confirmed by chromatography on Sephadex G-100; it is noteworthy that only ~25% of the C-glucan sample applied to the column was recovered. The S-glucan behaved very similarly to the C-glucan when examined on Sephadex G-200; however, an estimate of its molecular weight by determination of

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TABLE I

METHYLATION ANALYSIS OF *Rhizobium japonicum* GLUCANS

Alditol acetate derived from	T[a]	*C-glucan (mol %)*	*S-glucan (mol %)*
2,3,4,6-Tetra-*O*-methylglucose	1.00	9	9
2,4,6-Tri-*O*-methylglucose	2.04	56	42
2,3,4-Tri-*O*-methylglucose	2.54	27	39
2,4-Di-*O*-methylglucose	5.60	8	10

[a]Retention time of the alditol acetate derivative relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an ECNSSM column at 165° ($T_{1,0}$ = 8.9 min).

its reducing power gave a higher value, of ~12,000. Both glucans, but especially the S-glucan, exhibited a tendency to retrograde slowly; aqueous solutions were initially clear but became turbid and eventually formed precipitates after some days. The glucans were levorotatory: C-glucan, $[\alpha]_D$ −35°; S-glucan, $[\alpha]_D$ −27°.

The glucans were methylated and analyzed as the corresponding alditol acetates by g.l.c.–m.s.[2,3] (Table I). These results established that each of the polysaccharides consists of (1→3)-, (1→6)-, and (1→3)(1→6)-linked and non-reducing terminal glucopyranose components, but that their proportions in the two glucans are different. Whereas the S-glucan contains equal proportions of (1→3)- and (1→6)-linked residues, the C-glucan contains twice as many of the former as the latter.

The only anomeric signals in the ^{1}H-n.m.r. spectra of the two glucans were complex (δ 4.73 and 4.47), but established that β linkages of two types are present in each. However, the relative intensities of the two signals were different in the two glucans (Fig. 1); in the S-glucan, equal signal intensities were observed, whereas in the C-glucan, the intensity at δ 4.73 was twice that at δ 4.47. These observations agree with the results obtained by methylation analysis and correlate the signals at δ 4.73 and δ 4.47 with β-(1→3) and β-(1→6) linkages, respectively. While both anomeric-proton signals are highly complex, that at δ 4.73 exhibits greater multiplicity, indicating greater neighbouring-group influence on the β-(1→3) than on the β-(1→6) linkages.

The ^{13}C-n.m.r. spectra (Fig. 2) of the two glucans exhibited resonances characteristic of unsubstituted polysaccharides[4−8]. Thus, the regions about 61.6, 69.0, 73.9, 76.0, 85.2, and 103.5 p.p.m. may be assigned to C-6, C-4, C-2, C-5, C-3, and C-1, respectively, in a glucose residue. However, the number of signals in the regions assigned to C-1, C-3, C-4, and C-5 far exceed those observed previously in polysaccharides having definite repeating-units, and suggest that both the C- and S-glucans are highly branched and without defined repeating-units. In the S-glucan, there appear to be not fewer than six types of anomeric carbon atoms, two of which (signals at 103.85 and 103.65 p.p.m.) are dominant and may be assigned to the β-(1→6) and β-(1→3) linkages, respectively. At C-3, as many as eight residues are indicated, while the (1→6) and (1→3) linkages are further established by the reso-

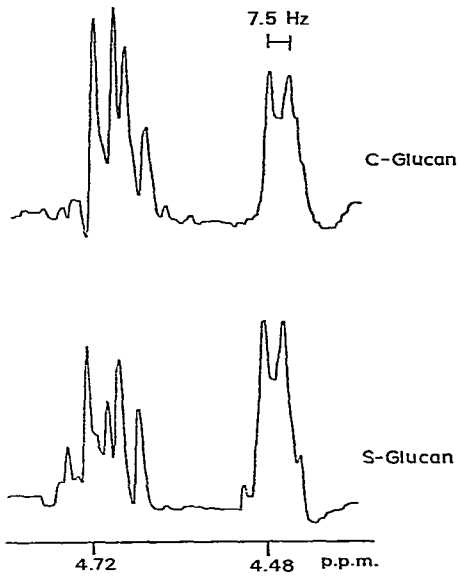

Fig. 1. The ^{1}H-n.m.r. spectra of the anomeric protons in the "C-" and "S-" glucans, measured at 80°. Convolution-difference methods were used to enhance resolution, with some distortion of the usual Lorentzian lineshape.

nances at 75.76 and 76.47 p.p.m. in the C-5 region. In the C-glucan, the six anomeric centres are observed, although in different proportions; the additional, intense resonance at 103.36 p.p.m. (of intensity equal to those at 103.85 and 103.65 p.p.m.) may be assigned to terminal, non-reducing residues. The ^{13}C spectra thus confirm considerable structural differences between the C- and S-glucans and establish the absence of defined repeating-units.

The methylation and n.m.r. analyses do not give information about the order in which residues are linked, and several alternative types of structure may be proposed that would lead to the same pattern of methylation products shown in Table I. However, it is possible to distinguish types of structure in which β-(1→6)-linked residues are in the main chain of the polysaccharide from those in which they are restricted to side chains, because they are the only residues susceptible to periodate oxidation. The S-glucan consumed the predicted amount of periodate [2 mol for each β-(1→6)-linked and each non-reducing terminal glucose residue] and, after sequential reduction and hydrolysis with dilute acid, was fragmented into small oligosaccharides. This indicated that, in the S-glucan, the periodate-resistant residues are not in one continuous chain but are interspersed with the β-(1→6)-linked residues. The C-glucan was not available in sufficient quantity for it to be examined in the same way.

Cellulose fibrils have been reported present in representative strains of all groups of rhizobia[9], and a glucan having predominantly β-(1→2)-linked residues and a small number of β-(1→3) and β-(1→6)-linked residues was detected in *R.*

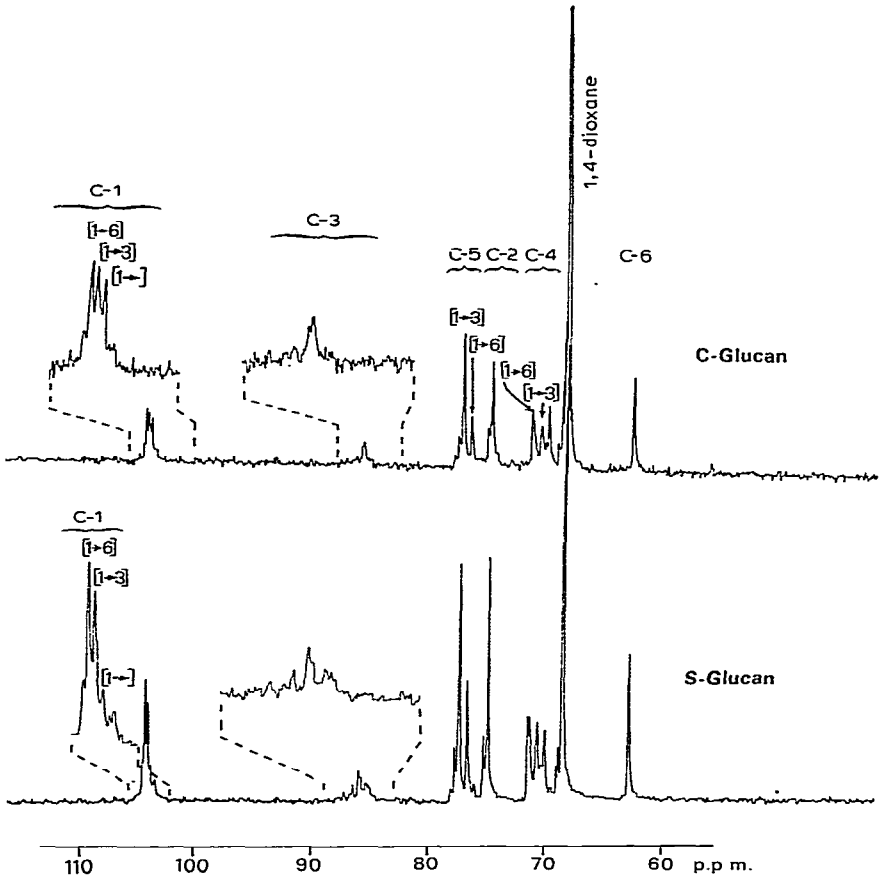

Fig. 2. The ^{13}C spectra of the "C-" and "S-" glucans for solutions in D_2O with 1,4-dioxane as internal reference. The chemical-shift regions characterizing specific carbon atoms are indicated by brackets. Expansions of the anomeric carbon atom (C-1) and C-3 are inset and show the considerable multiplicity of linkages in the two species. Some specific assignments of the linkages involved are indicated.

japonicum[10]; other unspecified glucans have been detected in *R. leguminosarum* and *R. lupini*[11]. However, the present polysaccharides are the first glucans consisting solely of β-(1→3) and β-(1→6) linkages to be observed in rhizobia. β-(1→3)-Glucans are extremely widespread in fungi, algae, and plants[12], but little is known of their distribution in bacteria.

The extracellular polysaccharides of rhizobia are the subject of considerable interest and speculation because they may play a role in the specificity of symbioses between *Rhizobium* species and their plant hosts. The currently favoured mechanism for this involves these polysaccharides and lectins from plant roots[13]. The possibility that the presently described glucans may be involved in this biological process is especially interesting because other glucans of similar, apparently simple, structures have been found to possess considerable biological activity[14]. It is particularly in-

triguing that a family of glucans very similar in structure to the present *R. japonicum* glucans, but ranging in molecular weight from 2,500 to >100,000, have been isolated from the soybean pathogen *Phytophthora megasperma* var. *sojae*; some of these fungal glucans have been demonstrated to elicit the formation of phytoalexins from soybeans, red kidney beans, and potatoes[15]. A yeast glucan of similar composition has the same elicitor action[16]. The present *R. japonicum* glucans have been examined for their ability to elicit the formation of phytoalexins in *Phaseolus vulgaris*, and the S-glucan, but not the C-glucan, was found to have such activity (I. A. M. Cruickshank, personal communication). It remains to be established whether or not these glucans from *R. japonicum* play a role in its symbiosis with soybeans.

EXPERIMENTAL

General methods. — The strain (3I1b71a) of *Rhizobium japonicum* and the general methods of analysis used have been described[1,17]. The oxidation of glucose (2 mg) from hydrolyzed, neutralized S-glucan by D-glucose oxidase (0.1 mg; Sigma, type II from *Aspergillus niger*) was performed in 2 mL of acetate buffer, pH 5.1, at 35°, with oxygen bubbled through the mixture; residual glucose was determined at intervals by the anthrone reaction.

Preparation of the glucans. — The organism was grown in two 10-L batches of a defined medium[17] in stirred, aerated fermentors (New Brunswick "Microferm") for 17 days. The cultures were centrifuged (1 h at 16,000*g*) and the clear supernatant solutions concentrated to one third of the original volume by evaporation under diminished pressure. Acetone (3 vol.) was added and the mixture kept for 3 days to allow the fine, non-gelatinous precipitate to settle; the precipitate was dissolved in water and dialyzed against flowing tap-water (2 days) and three changes of distilled water for another 2 days. The contents of the dialysis sacs had become turbid; these solutions were deionized with Zeokarb 225(H^+) resin, during which treatment the turbidity rapidly cleared to faint opalescence.

The deionized solution (1 L) was titrated from pH 2.8 to 7.0 with freshly prepared, filtered barium hydroxide solution to convert the rhamno-4-*O*-methylglucuronan into its barium salt. Solid sodium acetate trihydrate (4 g) was dissolved in the solution, acetone was then added, with stirring, to a final concentration of 63% (by volume), and the mixture was kept overnight at 4°. The flocculent, non-gelatinous precipitate (Fraction I) was removed by centrifugation and analyzed[17]; it contained 86% rhamno-4-*O*-methylglucuronan and 14% glucan. More acetone was added to the supernatant liquid to bring its concentration to 75% (by volume), when a further precipitate (Fraction II) containing 65% rhamno-4-*O*-methylglucuronan and 35% glucan was formed and recovered as before.

The acetone-rich supernatant liquid was free of acidic polysaccharide; it contained glucan and another constituent which upon methanolysis and trimethylsilylation gave a single peak in gas chromatograms, which indicated retention times similar to those of rhamnose on both OV-101 and OV-225 columns[1]. The material

responsible for this peak was extracted with chloroform and found by mass spectrometry (chemical ionization, with isobutane) to be a mixture of phthalic esters[18], of which di(ethylhexyl) phthalate was the main component (*m/e* 113, 149, 205, 279; $[M+1]^+$, 391). The glucan solution was evaporated under diminished pressure to remove the acetone and the phthalic esters removed by continuous extraction with chloroform. The solution was deionized with Zeokarb 225(H^+) resin and the glucan isolated by freeze-drying; yield of "supernatant" or "S-" glucan, 0.15 g, $[\alpha]_D$ −27° (*c* 0.4, water).

Fractions I and II were dissolved in water (100 mL), deionized with Zeokarb 225 (H^+), and the rhamno-4-*O*-methylglucuronan separated from the glucan by chromatography on a column (86 × 5 cm) of Sephadex G-200 (ref. 1). The glucan components in Fractions I and II were eluted at the same volume (K_{av} 0.89) and were combined. The glucan solution was dialyzed exhaustively against distilled water and the polysaccharide isolated by freeze-drying; yield of "column" or "C-" glucan, 70 mg; $[\alpha]_D$ −35° (*c* 4, water).

Approximate molecular weight. — The molecular weight of the C-glucan was re-determined chromatographically by applying a sample (3 mg in 1 mL) to a column (88 × 2.5 cm) of Sephadex C-100 Superfine Gel eluted with phosphate-buffered saline[1] by upward flow at 6.4 mL/h. The C-glucan was eluted at a larger volume (K_{av} 0.76) than the smallest available dextran standard (Dextran T10, Pharmacia) (M_w 9300; K_{av} 0.48), indicating that the C-glucan molecule was smaller than this. Only 25% of the glucan sample applied to the column was recovered; the remainder was not eluted in the void volume and was retained on the column[1].

The S-glucan was examined similarly on a column (88 × 5 cm) of Sephadex G-200 and its elution volume (K_{av} 0.89) found to be identical with that of the C-glucan on the same column; nothing was eluted at the void volume and only 10% of the sample applied was recovered. The reducing power[19] of the native S-glucan (0.7 mg sample) was equivalent to 1.38% of its weight of glucose, indicating a d.p. of 72 and a molecular weight of 11,740.

Methylation analysis. — Samples (10 mg) of the S- and C-glucans were dried *in vacuo* over phosphorus pentaoxide overnight at 60° and methylated[2], with addition, repeated after 2 h, of the methylsulphinylmethylide and methyl iodide reagents. The methylated glucans were analyzed as the alditol acetate derivatives[3].

Nuclear magnetic resonance spectra. — Samples of C-glucan (60 mg) and S-glucan (52 mg) were dissolved in deuterium oxide (0.5 mL) and dried over phosphorus pentaoxide; this process was repeated twice. The glucans were then redissolved in deuterium oxide (1.5 mL) and 1,4-dioxane (10 μL) added as the internal standard. The proton- and ^{13}C-n.m.r. spectra were measured at 270 and 67.89 MHz, respectively, with a Bruker HX-270 spectrometer. To resolve the anomeric region, the proton spectra were determined at 80° (ref. 20).

Periodate oxidation. — A sample (17 mg) of the S-glucan was treated with sodium periodate (10 mL, 0.05M) in the dark at 4°. The concentration of periodate was determined spectrophotometrically[21] at intervals (17, 24, and 40 h) and con-

sumption of oxidant found to be 0.91, 0.91, and 1.01 mol of periodate per glucose residue, respectively. Ethylene glycol (2 mL) was added and the mixture treated as described previously[1]. The periodate-degraded glucan (3 mg in 1 mL) was applied to a column (11 × 1 cm) of Bio-Gel P-2 (100–200 mesh; BioRad) operated at 60°. The column was eluted with water at 34 mL/h and the effluent monitored by the anthrone reaction. No polymeric material was detected in the void volume; three peaks having elution volumes corresponding to oligosaccharides with 2.5, 3.5, and 6 hexose residues were observed, in amounts equivalent to 43, 26, and 31% of the total carbohydrate material recovered.

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