Carbohydrate Research, 137 (1985) 167–171
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE STRUCTURE OF THE ACIDIC, EXOCELLULAR POLYSACCHARIDE PRODUCED BY *Rhizobium trifolii* BART A

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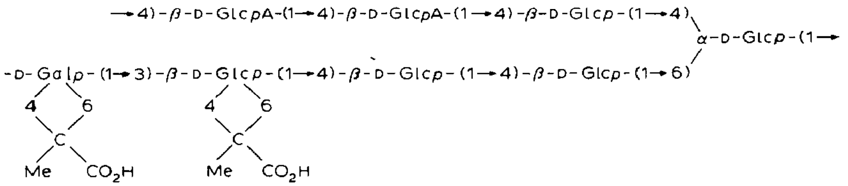
(Received June 19th, 1984; accepted for publication, August 24th, 1984)

ABSTRACT

The repeating unit of the acidic polysaccharide secreted by *Rhizobium trifolii* Bart A, a non-nodulating strain, is an octasaccharide containing *O*-acetyl and pyruvic acetal groups. The polysaccharide contains D-glucose, D-galactose, D-glucuronic acid, pyruvic acid, and acetic acid in the molar ratios 5:1:2:2:3. The glycosyl residues are all pyranoid and only the linkages between the repeating units are α. The repeating unit is different from that found in another non-nodulating strain but the same as that found in a nodulating strain of the same bacterial species, and the following structure is proposed.



INTRODUCTION

Rhizobium bacteria, which live symbiotically in the nodules on the roots of legume plants, have the ability to fix nitrogen. There is strong evidence which indicates that the acidic exopolysaccharides produced by these bacteria participate in the initial stages of recognition leading to host-legume nodulation through interaction with lectins in the root hairs[1]. Hence, structural variations would be expected in the polysaccharides participating in the specific host-symbiont selection. The structures of several acidic exopolysaccharides synthesised by *Rhizobia* have been elucidated, but more information is needed in order to obtain a clear answer to the specific role of these polysaccharides.

A structural relationship would be expected, especially for the polysaccharides produced by different strains of the same species. However, structural examination of the polysaccharides produced by four strains of *Rh. phaseoli* revealed variations, although there were certain features in common[2–5]. Even the

0008-6215/85/$ 03.30

polysaccharides produced by a non-nodulating strain[4] did not possess outstanding features compared to those of the strains having nodulating abilities. The variation of the structures within the nodulating[6-10] and non-nodulating[11] strains of *Rh. trifolii*, is even greater.

We now report on the structure of the acidic exopolysaccharide of *Rh. trifolii* Bart A, a non-nodulating strain.

EXPERIMENTAL

General methods. — The cultivation of *Rh. trifolii* Bart A and the preparation of the polysaccharide were as reported earlier[12]. The analytical procedures and chemical manipulations were all performed as described elsewhere[12-15], except when otherwise indicated. Acid hydrolysis was effected with 2M trifluoroacetic acid for 2 h at 120°.

Alditol acetates were analysed by g.l.c. on a Hewlett–Packard 5380 A gas chromatograph with a flame-ionisation detector and a glass column (300 × 0.2 cm) filled with 3% of OV-225 on 100/120 Supelcoport, programmed at 3°/min from 190→220°. Mass spectra were recorded on a Micromass 12F instrument, using a glass column filled with the OV-225 phase.

^{1}H-N.m.r. and proton-noise-decoupled ^{13}C-n.m.r. spectra were recorded for solutions in D_2O at room temperature with a Bruker 200-MHz F.t. spectrometer. The chemical shifts for the ^{13}C signals were assigned relative to that of acetone (30.5 p.p.m. downfield from the signal for Me_4Si) and for the ^{1}H signals relative to that of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

Methylation analysis. — To obtain a solution of the polysaccharide in methyl sulfoxide, the mixture had to be sonicated occasionally for 2 weeks. The Hakomori procedure[16] was used, but with the potassium anion[17] since this made it easier to obtain a fully methylated product. Prolongation of the reaction time of the carbanion to 48 h also improved the result. A part of the methylated polysaccharide was hydrolysed, and the products were acetylated and submitted to g.l.c. and g.l.c.–m.s.[15].

Carboxyl-reduction. — A solution of the methylated polysaccharide (5 mg) in dry tetrahydrofuran (1.5 mL) was added to a stirred suspension of lithium aluminium hydride (30 mg) in the same solvent (2 mL). The slurry was stirred for 24 h at room temperature, water was added, and the mixture was extracted with dichloromethane (3 × 5 mL). The material extracted was hydrolysed, and the products were acetylated and examined by g.l.c. and g.l.c.–m.s.

A portion of the methylated, carboxyl-reduced polysaccharide was re-methylated.

RESULTS AND DISCUSSION

The polysaccharide was isolated from 6-day-old cultures, since it was anticipated that its composition at this stage had become constant[18]. The acidic polysaccharide was isolated by application in sequence of ethanol-precipitation, selective precipitation with cetyltrimethylammonium bromide, ethanol-precipitation, dialysis, and freeze-drying. The product had a low solubility in water and methyl sulfoxide but, in order to prevent loss of labile constituents, no further steps were performed to make it more soluble.

The polysaccharide contained glucose, galactose, and glucuronic acid all in the D configuration[12]. The products in an acid hydrolysate of the polysaccharide were reduced, acetylated, and then subjected to g.l.c. and g.l.c.–m.s. The results indicated D-glucose and D-galactose to be present in the polysaccharide in the molar ratio 5:1. The same ratio was found by methylation analysis of the polysaccharide (Table I, column A). After enzymic determination of pyruvic acid[19] and colorimetric determination of D-glucuronic[20] and acetic acid[21], the total composition of the polysaccharide was shown to be D-glucose, D-galactose, D-glucuronic acid, pyruvic acid, and acetic acid in the molar ratios 5:1:2:2:3. Methylation analysis of the polysaccharide, with g.l.c. and g.l.c.–m.s. of the partially methylated alditol acetates (Table I, column A), showed that it contained D-glucopyranosyl residues linked through O-4, O-4,6, and O-3,4,6, and D-galactopyranosyl residues linked through O-4,6. Analysis of the polysaccharide after methylation and carboxyl-reduction showed (Table I, column B) an increase in the proportion of 2,3-di-*O*-methyl-D-glucose, demonstrating that the glucopyranosyluronic acid residues were linked through O-4. This finding was confirmed by analysis of the re-methylated material (Table I, column C), where an increase in the proportion of 2,3,6-tri-*O*-methyl-D-glucose was observed. The proportions of 2,3-di-*O*-methyl-D-glucose of B and 2,3,6-tri-*O*-methyl-D-glucose of C were lower than the theoretical values for the postulated repeating-unit, due to the usual difficulties in effecting quantitative carboxyl-reduction. But the colorimetric determination of D-glucuronic acid, which gave the theoretical value, and the identification of oligosaccharides containing two contiguous D-glucosyluronic acid residues[13] indicated that the repeating unit contained two D-glucosyluronic acid residues. The D-galactopyranosyl residue was terminal and 4,6-substituted with pyruvic acid acetal, as shown for the pyruvic acid-containing oligosaccharides[14]. The O-3,4,6-linked D-glucopyranosyl residue was adjacent to the terminal D-galactopyranosyl residue and probably carried a 4,6-pyruvic acid acetal. In the isolated oligosaccharides[14,15], the pyruvic acid acetal group was lost; on methylation analysis, the relevant glucosyl residue appeared as 2,4,6-tri-*O*-methyl-D-glucose. The quantity of pyruvic acid, determined enzymically, accounts for 2 mol of pyruvic acid per repeating unit, but it was not possible to isolate oligosaccharides having pyruvic acid linked to the glucosyl residues. The O-4,6-linked D-glucopyranosyl residue was the branch-point of the backbone of the polysaccharide, and the O-4-linked D-glucopyranosyl residues were present in both side-chains and the backbone, as shown by the structure of the oligosaccharides.

The sequence of the sugar residues was ascertained by determination of the structure of the neutral[15], glucuronic acid-containing[13], and pyruvic acid-containing

TABLE I

METHYLATION ANALYSIS OF ORIGINAL AND CHEMICALLY MODIFIED *Rh. trifolii* BART A EXOCELLULAR POLYSACCHARIDES

Methylated sugar[a]	T[b]	*Mole %*[c]			
		A	*B*	*C*	*D*
2,3,4,6-Glc	1.00				6
2,3,4,6-Gal	1.08				9
2,4,6-Glc	1.39				11
2,3,6-Glc	1.57	50	40	61	53
2,3-Glc	2.12	16	32	13	18
2,3-Gal	2.15	17	13	12	3
2-Glc	2.60	18	14	14	

[a] 2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, *etc.* [b] Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol [c] Polysaccharides (for details, see text) A, original; B, methylated and reduced; C, methylated, reduced, and re-methylated; D, partially degraded polysaccharide

oligosaccharides[14] isolated from the polysaccharide of the bacterial strain.

The oligosaccharides isolated were combinations of all the sugar sequences possible in the repeating unit. On the basis of enzymic methods, the linkages were shown to be β. It was not possible to isolate an oligosaccharide which contained the branch-point glucosyl residue and the adjacent glucosyluronic acid residue, and ^{1}H- and ^{13}C-n.m.r. spectroscopy were used to determine the linkage between these units. Due to the low solubility of the polysaccharide, the partially degraded polymer formed by the preparation of the oligosaccharides[14] was used. The polysaccharide (0.5%) was treated (2 h) with boiling trifluoroacetic acid (0.5M), and the hydrolysate was passed through an Amicon type PM-10 membrane (10,000 mol. wt. cut-off). When the high-molecular-weight fraction was submitted to gel filtration, a single peak was eluted from a column of Sepharose 4B after the void volume. Analysis of this polysaccharide revealed that ~90% of the original content of both pyruvic acid and acetic acid had been removed and that the content of glucuronic acid had been increased from 22% to 36%, indicating a loss of neutral constituents. Methylation analysis (Table I, column D) showed that the backbone, which contained the inter-repeating-unit linkage, was intact, but that the side chains were either intact or totally removed as the proportion of 2,3,4,6-tetra-*O*-methyl-D-glucose was low. Most of the terminal residues of the side chains had lost their pyruvic acid acetal groups, as revealed by the formation of 2,3,4,6-tetra-*O*-methyl-D-galactose. The pyruvic acid acetal groups were totally removed from the glucosyl residues, giving rise to 2,4,6-tri-*O*-methyl-D-glucose in a proportion equal to the sum of those of the two galactose derivatives, which represent intact side-chains as far as the sugar residues are concerned.

The aim of the n.m.r. study of the degraded polymer was to determine the configuration of the linkage between the repeating units, and hence attention was paid to the anomeric regions of the spectra. The ^{1}H-n.m.r. spectrum contained multiple signals for H-1β, but only one signal for H-1α at δ 5.23 which was insufficiently resolved to permit measurements of the coupling constant. The ^{13}C proton-noise-decoupled spectrum contained several signals for C-1β (δ 102.3–104.17), but only one for C-1α at δ 100.11.

These data clearly show that the polymer contains an α linkage in addition to the β linkages identified by enzymic methods, and it is concluded that it is the inter-unit linkage which is α.

The results of this investigation, together with those of the structural determination of the oligosaccharides[13–15] isolated from the same polysaccharide, lead to the conclusion that the polysaccharide produced by *Rhizobium trifolii* Bart A, a non-nodulating strain, contains the repeating unit proposed in the Abstract. This unit is the same as that found in the polysaccharide of a nodulating strain of the same species[8], but slightly different from the unit found in another nodulating strain[10]; it lacks the terminal, pyruvylated galactosyl group of the side chain. This might be due to different handling of the polysaccharide.

ACKNOWLEDGMENT

The n.m.r. analyses were carried out at the National Laboratory for N.m.r. Analysis at the University of Oslo, which is funded by The Norwegian Research Council for Science and the Humanities.

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