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THE STRUCTURE OF THE NEUTRAL POLYSACCHARIDE GUM SECRETED BY *Rhizobium* STRAIN CB744

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ABSTRACT

A previous investigation[1] of the structure of the extracellular polysaccharide gum from the nitrogen-fixing *Rhizobium* strain CB744 (a member of the slow-growing Cowpea group) indicated that there were two β-(1→4)-linked D-glucopyranosyl residues for each α-(1→4)-linked D-mannopyranosyl residue, and that each mannose was substituted at O-6 by a β-D-galactopyranosyl residue having 71% of the galactose present as 4-*O*-methylgalactose. The present study shows that, although the gum appeared to have a simple tetrasaccharide repeating unit, it is composed of two closely associated components. One is a (1→4)-linked α-D-mannan substituted at each O-6 by a β-D-galactopyranosyl residue (71% 4-*O*-methylated). The second component is a (1→4)-linked β-D-glucan. The existence of the two polysaccharides was established by separation of the β-D-galactosidase-treated gum on a column of concanavalin A–Sepharose 4B. The D configurations were determined and the anomeric attribution of the linkages confirmed by the use of enzymes. The interaction between the two gum components is discussed.

INTRODUCTION

The ability of *Rhizobium* bacteria to fix nitrogen by their symbiotic interaction with leguminous plants is of great importance in agriculture. Members of the Cowpea group are typical of the slow-growing *Rhizobium* species in that they produce extracellular polysaccharides that vary greatly in composition within species[2,3]. In contrast, fast-growing rhizobia secrete polysaccharides that do not vary greatly in composition. A recent example of this is an investigation[4] in which three strains of *Rhizobium leguminosarum* and one strain of *Rhizobium trifolii* were shown to produce the same extracellular polysaccharide, which was similar in structure[5] to the polysaccharide from a strain of *Rhizobium phaseoli*.

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Our initial investigation[1] of the structure of the extracellular polysaccharide gum from the slow-growing *Rhizobium* sp. strain CB744, a member of the Cowpea group, confirmed the diversity of structure of the slow-growing group. The polysaccharide gum was found to be a branched structure composed of neutral sugars.

For every two β-(1→4)-linked glucopyranosyl residues there is one α-(1→4)-linked mannopyranosyl residue. The side chain consists of a single D-galactopyranosyl group intermittently methylated at O-4. This group is linked to O-6 of the mannopyranosyl residues in the backbone.

We report here further studies on the polysaccharide gum produced by *Rhizobium* sp. strain CB744 that show it to be a mixture of two components.

EXPERIMENTAL

General. — *Rhizobium* sp. strain CB744 was maintained and the polysaccharide gum isolated as previously described[1]. For further purification, an aqueous solution of polysaccharide was partitioned with 80% aqueous phenol. Analysis of the monosaccharide components shows a change in composition which indicates that the polysaccharide undergoes partial degradation during phenol treatment. Unless otherwise stated, therefore, the results were obtained by using polysaccharide that had not been treated with phenol. Total acid hydrolysis of the enzyme-treated polysaccharides, and of the components separated by affinity chromatography on concanavalin A–Sepharose, was accomplished by boiling under reflux in M sulphuric acid for 16 h. Details of general methods and gas–liquid chromatography (g.l.c.) of the peracetylated alditols have been described[1].

Materials. — Electrophoretically purified cellulase III from *Thermoascus aurantiacus* was a gift from Dr. M. G. Shepherd, University of Otago, and crude cellulase type II from *Aspergillus niger* was obtained from Sigma Chemical Co. Dr. B. V. McCleary (Biological and Chemical Research Institute, N.S.W.) kindly donated the *Bacillus subtilis* β-D-mannanase. β-D-Galactosidase from bovine liver was free from α-D-galactosidase activity as tested with methyl α-D-galactopyranoside as substrate. This and other enzymes and nucleotides were purchased from Sigma Chemical Co. Concanavalin A–Sepharose 4B, Sephadex G-200, and Sephacryl S-200 were purchased from Pharmacia.

Treatment with cellulase. — The polysaccharide gum (10 mg) was dissolved in 0.1M sodium acetate buffer, pH 4.0 (1 mL). This was incubated with a solution (1 mL) containing 25 μg of crude *A. niger* cellulase for 48 h at 40° as described by Hurst *et al.*[6], and then heated for 5 min at 100° to inactivate the enzyme. An aliquot of the hydrolysate was subjected to paper chromatography (p.c.) and the remainder of the enzyme hydrolysate was dialysed for 4 days against distilled water. The diffusible material was evaporated to low volume (50 mL) and divided into two equal aliquots. One portion was reduced and acetylated for g.l.c. analysis, while the other was successively hydrolysed for 16 h with M sulphuric acid, made neutral, reduced, and acetylated for g.l.c. analysis. Blank hydrolyses were conducted simul-

taneously. The procedure was repeated by using a partially purified cellulase III from *T. aurantiacus*.

Treatment with β-D-galactosidase. — A 1.0% solution of polysaccharide gum in 0.1M sodium phosphate buffer (pH 7.0) was prepared. This solution (4 mL) was incubated with a freshly prepared solution (0.1 mL) containing 0.3 mg.mL^{-1} of β-D-galactosidase in 0.1M disodium hydrogenphosphate–potassium dihydrogenphosphate (pH 7.0) for 48 h at 37° (ref. 7). An aliquot of the hydrolysate was subjected to p.c., and the remainder of the enzyme hydrolysate dialysed for 4 days against distilled water. The diffusible material was evaporated to low volume (50 mL) and divided into two aliquots. One was reduced and acetylated, the other was successively hydrolysed, reduced, and acetylated. Both portions were subsequently examined by g.l.c. The non-diffusible portion was hydrolysed, reduced, and acetylated for g.l.c. analysis. Other substrates subjected to this procedure were guar gum and methyl α-D-galactopyranoside. In addition, controls were performed with substrate excluded and with enzyme excluded.

Treatment with β-D-mannanase. — *B. subtilis* β-D-mannanase[8] activity was assayed[9] by using α-D-galacto-β-D-mannan dyed with Remazol Brilliant Blue. Partially acid-hydrolysed[1] polysaccharide (15 mg) was dissolved in 0.3M acetate buffer (pH 5.1, 2 mL), β-D-mannanase (0.5 mL, 0.1 μkat.mL^{-1}) added, and the resulting mixture incubated in an oscillating bath for 24 h at 40°. The mixture was dialysed against distilled water for 3 days and the dialysis water concentrated for identification of any low-molecular-weight substances produced. This low-molecular-weight material was successively hydrolysed, reduced, and acetylated for detection of sugars by g.l.c. The non-diffusible material was divided into two aliquots. One portion was hydrolysed, reduced, and acetylated for g.l.c. analysis. To the other, α-D-galacto-β-D-mannan dyed with Remazol Brilliant Blue (0.5 mL, 0.75%) was added and the mixture was again incubated as before.

Enzymic assay of D-mannose and D-glucose. — A total acid-hydrolysate was phosphorylated with hexokinase in the presence of adenosine triphosphate (ATP) as described by Gawehn[10]. D-Glucose 6-phosphate thus produced was estimated quantitatively by using D-glucose-6-phosphate dehydrogenase and nicotinamide adenine dinucleotide phosphate ($NADP^+$). Absence of D-fructose was confirmed by the lack of reaction after addition of phosphoglucoisomerase, and D-mannose was then assayed with phosphomannoisomerase.

Chromatography on concanavalin A–Sepharose. — A modified version of the procedure of Lloyd[11] was used. A column (22 × 0.9 cm) of concanavalin A–Sepharose 4B was washed (6 volumes) with 0.9% sodium chloride in 18mM sodium phosphate buffer (pH 7.45) and a solution (1 mL) containing the phenol- and β-D-galactosidase-treated polysaccharide gum (3.9 mg) was applied to the column. Elution of the column with sodium chloride–phosphate buffer (100 mL) was followed by linear gradient-elution with sodium chloride–phosphate buffer (200 mL) and sodium chloride–phosphate buffer (200 mL) containing 0.05M methyl α-D-mannopyranoside. After estimation of the optical density at 420 nm, appropriate frac-

tions were combined, dialysed against distilled water for 5 days, dried, and weighed. The yield of the polysaccharide eluted first was 1.6 mg and the second was 1.5 mg. Samples were then hydrolysed, reduced, and acetylated for g.l.c. analysis.

RESULTS AND DISCUSSION

Previous work[1] showed the extracellular polysaccharide gum from *Rhizobium* sp. strain CB744 was composed of galactose, 4-*O*-methyl-galactose, mannose, and glucose in the molar ratios of 1:2.5:3.5:7.0. After incubation of the polysaccharide with a commercial preparation of cellulase isolated from *A. niger*, the hydrolysate was dialysed. A total acid-hydrolysate of the non-diffusible fraction was shown (p.c., g.l.c.) to contain galactose, 4-*O*-methylgalactose, and mannose in the molar ratios of 1:2.5:3.5. The diffusible fraction was shown to contain glucose (p.c., g.l.c.). After acid hydrolysis, no molar increase in glucose was detected. As no hydrolytic action was observed when amylose was used as the substrate for this enzyme preparation, it follows that D-glucose must be in the β configuration, in agreement with the results[1] of ^{13}C-n.m.r. spectroscopy and oxidation of the native polysaccharide by chromium trioxide.

Methylation-analysis data, in combination with ^{13}C-n.m.r. and oxidation by chromium trioxide demonstrated[1] that the polysaccharide has a branched structure having two β-(1→4)-linked glucopyranosyl residues for each α-(1→4)-linked mannopyranosyl residue. The side chain consists of single β-D-galactopyranosyl groups (71% of which are methylated at O-4) attached to O-6 of the mannopyranosyl residues. As the treatment with crude cellulase results in the total removal of glucose, leaving high-molecular-weight material containing galactose, 4-*O*-methylgalactose, and mannose, the polysaccharide cannot have a regular tetrasaccharide repeating-unit.

When the partially purified cellulase from *T. aurantiacus* was used as already described, the non-diffusible fraction was shown (p.c. and g.l.c. of alditol acetates, after total acid hydrolysis) to contain galactose, 4-*O*-methylgalactose, mannose, and glucose in the molar ratios 1:2.5:3.5:0.3. This is again consistent with the D-glucose being in the β configuration and with the absence of a tetrasaccharide repeating-unit.

When the total polysaccharide was incubated with β-D-galactosidase, p.c. of an aliquot of the mixture demonstrated that galactose was released. Low-molecular-weight material was removed by dialysis and shown (p.c., g.l.c.) to contain only galactose. After further acid hydrolysis of the low-molecular-weight fraction, no change in g.l.c. pattern was observed. Complete acid hydrolysis showed (p.c., g.l.c.) the high-molecular-weight fraction to contain 4-*O*-methylgalactose, mannose, and glucose in the molar ratios of 1:1.4:2.8. Galactose was therefore completely removed, and no other sugar accompanied its removal. The action of β-D-galactosidase confirms the findings of the methylation analysis[1] that D-galactose is in the terminal position. The specificity of the enzyme[15] demonstrates that all of the

galactose is present as the D enantiomer and in the β configuration. 4-*O*-Methylgalactose was not removed by the galactosidase, which is consistent with the previous finding[12] that substitution of O-4 inhibits the enzyme action.

After removal of the galactose and 4-*O*-methylgalactose residues by partial acid hydrolysis[1], the remaining polymeric material was incubated with β-D-mannanase, with subsequent dialysis. No material of low-molecular-weight was detected. The high-molecular-weight material was hydrolysed and converted into alditol acetates for g.l.c. analysis, which showed mannose and glucose in the ratio of 1:2.0. That the enzyme was active throughout the incubation was demonstrated by adding Remazol Brilliant Blue-dyed α-D-galacto-β-D-mannan to an aliquot at the end of the experiment. The results of this experiment are consistent with the previous investigation[1], which indicated that mannopyranose exists in the α configuration.

Enzymic assay of sugars in the total acid hydrolysate of the polysaccharide gum by phosphorylation and reduction with $NADP^+$ confirmed that the molar ratio of mannopyranose to glucopyranose was 1:2.0. Moreover mannopyranose and glucopyranose could not be detected on completion of the assay, demonstrating that both sugars are exclusively D-enantiomers.

It is concluded from the results of the present work and the previous investigation[1] that the polysaccharide gum consists of two β-(1→4)-linked D-glucopyranosyl residues for each α-(1→4)-linked D-mannopyranosyl residue and each mannopyranosyl residue is substituted at O-6 by either a β-D-galactopyranosyl or a 4-*O*-methyl-β-D-galactopyranosyl group. In spite of the integral molar-ratio of the components, the conclusion reached in the preliminary investigation that the polysaccharide is composed of a regular tetrasaccharide repeating-unit has not been substantiated.

In the absence of a regular repeating-unit, it is possible, although unlikely, that the polysaccharide exists as a mixed polymer having blocks of β-D-galactosyl-substituted α-D-mannan interspersed with blocks of β-D-glucan. Alternatively, the gum may be a mixture of two components. In support of the latter idea is the frequency with which discrete β-D-glucans have been isolated from rhizobial polysaccharide gums[13–20]. The separation of two components would prove the latter hypothesis. Attempts to separate the polysaccharide gum by gel filtration using Sephadex G-200 (ref. 14) and Sephacryl S-200, by ultracentrifugation[21], and by electrophoresis[22] were unsuccessful. After treatment with β-D-galactosidase, the polysaccharide gum was separated into two components by affinity chromatography with concanavalin A–Sepharose 4B (Fig. 1). G.l.c. analysis of the alditol acetates of the acid hydrolysates showed that the first peak eluted consisted entirely of glucose and the second peak, eluted by methyl α-D-mannopyranoside solution, contained 4-*O*-methylgalactose and mannose in the molar ratio of 1:1.46. Therefore, it was concluded that the first peak was β-D-glucan (77% yield) and the second peak was a mannan having the unusual α-D configuration and with 4-*O*-methyl-β-D-galactosyl side branches (80% yield). Attempts to fractionate the

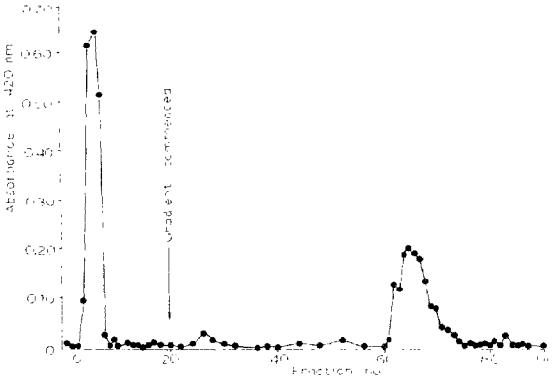


Fig. 1. Separation of β-D-galactosidase-treated polysaccharide on concanavalin A–Sepharose 4 B

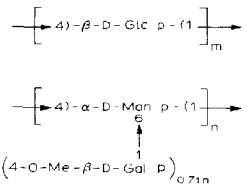
polysaccharide on concanavalin A–Sepharose 4B without prior treatment with β-D-galactosidase were unsuccessful.

The binding of an α-D-mannopyranosyl residue to concanavalin A occurs when the mannose is at either a non-reducing terminal position[24] or in an internal position substituted at O-2[24,25]. Both α-D-mannans[11,26] and α-D-glucans[11,27] have been shown to bind to concanavalin A–Sepharose, but these were highly branched structures. Retardation of a linear α-D-mannan is unusual, and may reflect a relatively low degree of polymerisation. The separation may also have been aided by the gel-filtration properties of Sepharose 4 B. Also of interest is the apparent solubility of the (1→4)-β-D-glucan. This could be explained in part if this component is oligomeric in nature, or if it contains some as-yet-unrecognised structural feature. The incomplete hydrolysis by purified cellulase may be relevant in this regard. A minor unexpected component could well escape recognition in ^{13}C-n.m.r. spectra of the native material.

The results of the affinity-chromatography experiments suggest that the D-mannopyranosyl residues have the α and the D-glucopyranosyl residues the β configuration. This interpretation supports the ^{13}C-n.m.r. assignments[1] and confirms the findings of oxidation by chromium trioxide, hydrolysis by cellulase, and the unsuccessful attempt at hydrolysis by β-D-mannanase.

More important, affinity chromatography shows that the modified gum consists of two polysaccharides (Scheme 1). The essentially exact whole-number ratio of glucose to mannose found in the two components suggests that their biosynthesis is under relatively fine control.

The (1→4)-β-D-glucan found in the present work appears to be distinctly different from previously reported *Rhizobium* glucans[14-16,18,19,28] as it is a major



Scheme 1

component of the mixture of extracellular polysaccharides. Furthermore, a number of reasons suggest that it is not a conventional cellulose molecule. These include the inability of the partially purified cellulase to hydrolyse it completely, and its apparent "solubility" after separation from the 4-*O*-methylgalactose-substituted mannan. In addition, its interaction with the β-D-galactose-substituted α-D-mannan is sufficiently strong that it is difficult to separate the two components. This interaction is indicated by the lack of separation on electrophoresis in borate buffer[22], and the generally "gummy" nature of the native material, together with the qualitative observations on viscosity noted later. The behaviour of the two components on affinity chromatography supplies additional evidence: retardation of the α-D-mannan shows either inclusion in the gel and hence a low molecular weight, or a sufficiently high proportion of non-reducing, terminal residues for interaction with the concanavalin A, and again a relatively low degree of polymerisation. Although the molecular weight of the substituted mannan before treatment with β-D-galactosidase would only be higher in the ratio of 7:6, the presence of the galactosyl substituent, with its presumed blocking of the interaction with concanavalin A, causes the glucan and mannan chains to be excluded from the gel, with an apparent molecular weight in excess of 10^6.

It is interesting to speculate on how the two components that make up the rhizobial gum interact strongly enough to act as one polysaccharide system. Conventional galactomannans, in which the mannan is β-(1→4)-linked are able to interact with xanthan gum to form a gel. Rees and coworkers[29] have explained this interaction in terms of "smooth" regions of the galactomannan interacting with the regular xanthan structure, whereas the galactose-substituted region does not participate in the interaction. Later, McCleary[30] demonstrated that alternately substituted galactomannan interacts strongly enough with xanthan to give a gel, but the galactose sidearms are apparently not involved in the interaction.

Unlike xanthan gum, where the (1→4)-β-D-glucan is part of a larger molecule[25,31], the (1→4)-β-D-glucan in the present case is a separate molecule interacting with a β-D-galactose-substituted α-D-mannan. The α-(1→4) linkage of the mannan backbone in the rhizobial polysaccharide requires it to adopt a conformation very different from that of a (1→4)-β-D-mannan. It is assumed that it will be analogous to amylose, and therefore be a "compressed spring" helix. However, because there is a β-D-galactopyranosyl substituent on every mannopyranosyl residue

of the α-D-mannan, the galactopyranosyl and 4-*O*-methylgalactopyranosyl sidearms of the β-galacto-α-mannan are involved in the interaction with the glucan. In support of this idea is the observation that removal of galactopyranose and 4-*O*-methylgalactopyranose residues by partial acid hydrolysis results in a marked decrease in solubility, and the more selective, enzymic removal of galactose also caused a decrease in viscosity. That the unsubstituted D-galactose residues are involved is also shown by the separation of the two components on concanavalin A–Sepharose 4B after, but not before, their removal.

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