Carbohydrate Research, 110 (1982) 267–276
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

CAPSULAR AND EXTRACELLULAR POLYSACCHARIDES FROM *Rhizobium* MICROSYMBIONTS OF *Acacia decurrens*

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(Received May 14th, 1982; accepted for publication, June 7th, 1982)

ABSTRACT

The capsular polysaccharide produced by a *Rhizobium* isolated from a root nodule of *Acacia decurrens* is composed of 3-*O*-methyl-L-rhamnose:L-rhamnose:D-mannose:D-glucose:D-galacturonic acid in the molar ratios of ~1:2:2:4:1. The extracellular polysaccharide is similarly constituted. Structural analyses indicate a decasaccharide repeating-unit in which the L-rhamnosyl groups occur as single-unit side-chains. The 3-*O*-methyl-L-rhamnosyl and one of the α-L-rhamnosyl groups are (1→6)-linked to two of the D-glucosyl residues. The other α-L-rhamnosyl group is (1→4)-linked to the D-galacturonic acid residue. The main-chain residues are all (1→3)-linked, and are partially identified as -(1→3)-α-D-Gal*p*A-(1→3)-α-D-Man*p*-(1→3)-α-D-Glc*p*-(1→3)-.

INTRODUCTION

An earlier report[1] described the production, isolation, and compositional analysis of a capsular polysaccharide produced by rhizobial microsymbionts of *Acacia decurrens* (black wattle). Although native to Australia, the tree is grown in Hawaii as an ornamental[2]. Our attention was directed to the polysaccharide, because it had been found to contain both L-rhamnose and its 3-methyl ether. Analysis[1] of the monosaccharide composition suggested a decasaccharide repeating-unit composed of 3-*O*-methyl-L-rhamnose:L-rhamnose:D-mannose:D-glucose:D-galacturonic acid in the molar ratios of ~1:2:2:4:1. As our analytical studies proceeded, a number of unusual, structural features became apparent: (*a*) all of the 6-deoxyhexose residues occur as (nonreducing) end groups, (*b*) the D-galactopyranosyluronic acid residue is a point of branching, and (*c*) the predominant linkage-type is (1→3).

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RESULTS AND DISCUSSION

Polysaccharides (PS) formed by *Rhizobium* strains originally isolated from root nodules of *A. decurrens* are all of the capsular type when the strains are grown on yeast extract, D-mannitol, and soil extract (YMS)[1]; *i.e.*, they can only be obtained free from cells after treatment with dilute alkali (0.1M KOH). Both an extracellular (EPS) and a capsular polysaccharide (CPS) are formed in synthetic medium. No pyruvic acetal or *O*-acyl substituents could be detected in the cell-bound CPS or in the EPS. Table I lists the sugar components of the CPS from strain 1B3 and related strains. All six isolates produced similar polysaccharides in which the molar ratios of total L-rhamnose : D-mannose : D-glucose : D-galactose is ~3 : 2 : 4 : 1.

Subsequent studies were conducted with strain 1B3. Shorter periods of hydrolysis gave lessened proportions of D-mannose concomitant with an increase in aldobiouronic acid, instead of free D-galacturonic acid. This result suggested that a portion of the D-mannosyl residues was linked to D-galacturonic acid. Reduction of the carboxyl groups[4] in the CPS and EPS converted the D-galactosyluronic into D-galactosyl residues. The reduced polysaccharides were readily hydrolyzed, and the neutral sugar components in the hydrolyzate were quantitated by gas–liquid chromatography (g.l.c.) as their derived per-*O*-acetylaldononitriles[5] (PAAN), to give the molar percentages of components listed in Table II. These percentages correspond to molar ratios of 3-*O*-methyl-L-rhamnose : L-rhamnose : D-mannose : D-glucose : D-galacturonic acid of 1 : 2 : 2 : 4 : 1.

Methanolysis of the CPS, followed by reduction of the uronic acid methyl ester, additional methanolysis, and analysis as the per(trimethylsilyl) (Me_3Si) ethers of the methyl glycosides confirmed these results. Even though the total proportion of 6-deoxyhexose remains constant, the EPS contains relatively less 3-*O*-methyl-L-

TABLE I

PERCENTAGE SUGAR COMPOSITION[a] OF CAPSULAR POLYSACCHARIDES[b] FROM NITRAGIN STRAINS AND A RELATED RHIZOBIUM

Rhizobium *strain*	*Sugar component* 3-O-*Me*-L-*Rha*	L-*Rha*	D-*Man*	D-*Glc*	D-*GalA*
1B3	10	18	24	37	11
1B4	7	21	23	37	12
IB5	9	18	25	40	8
1B6	6	22	24	36	12
1B7	9	20	23	38	10
USDA 3001[c]	7	25	23	36	9

[a]Analyzed as Me_3Si derivatives of methyl glycosides in reduced ($NaBH_4$) mixtures of methanolysis products (see Experimental section). By this procedure, D-GalA was analyzed as D-Gal. [b]Produced on YMS medium. [c]USDA-Beltsville *Rhizobium* Culture Collection. Strain originated in Brazil from root nodules of *A. decurrens*. Exopolysaccharide produced on Owen's medium C[3].

TABLE II

PERCENTAGE SUGAR COMPOSITIONS OF NATIVE AND SMITH-DEGRADED POLYSACCHARIDES OF *Rhizobium* sp. 1B3

Sugar component	*Capsular polysaccharides*				*Extracellular polysaccharides*			
	Native			*Degraded*	*Native*			*Degraded*
	YMS medium		*Owen's medium C*[a]	*YMS medium*	*Owen's medium C*			
	PAAN[b]	*Me$_3$Si*	*Me$_3$Si*	*PAAN*	*PAAN*	*Me$_3$Si*	*Me$_3$Si*[c]	*PAAN*
3-*O*-Me-L-Rha	7	9	7	15	5	4	8	11
L-Rha	15	18	17	—	23	23	25	1
D-Man	23	24	23	28	22	25	22	25
D-Glc	44	39	45	43	36	40	38	50
D-GalA	11	10	8	14	14	8	7	13

[a]Medium[3] contained Difco casamino Acids (2 g/L) and mixed carbon source of D-mannitol (8 g/L) and K gluconate (2 g/L). [b]Hydrolyzates of carboxyl-reduced polysaccharides were analyzed by g.l.c. as PAAN derivatives; Me$_3$Si derivatives were used to analyze methanolysis products, as in Table I. [c]Medium modified, in this experiment only, to contain 1 g of casamino acids/L, 9 g of D-mannitol/L, and 1 g of K gluconate/L.

TABLE III

METHYLATION ANALYSES OF CARBOXYL-REDUCED AND SMITH-DEGRADED EXTRACELLULAR AND CAPSULAR POLYSACCHARIDES OF *Rhizobium* sp. 1B3

Methylated sugar[a]	*T*[b]	*Mol%*						
		Capsular (YMS medium)		*Extracellular (Owen's medium C)*				
		Intact	*Degraded*	*Intact*			*Degraded*	
2,3,4-Rha	0.47	11[e]	13[c,d]	37[e]	24[f]	19[c]	11[e]	9
2,4,6-Glc	1.44	27	29	25	25	26	35	39[f]
2,4,6-Man	1.65	24	26	25	20	21	26	23
2,4,6-Gal	1.71	—	16	—	—	—	25	22
2,6-Gal	2.45	12	—	9	15	17	—	—
2,4-Glc	2.56	26	16	4	16	17	3	7

[a]2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, etc. [b]G.l.c. retention time of the corresponding per-*O*-acetylated aldononitrile relative to 5-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-gluconononitrile. [c]Formolysis preceded hydrolysis. [d]Carboxyl-reduced *after* Smith degradation. [e]Brief formolysis[7] (2 h) only. [f]No formolysis, but hydrolysis with sulfuric acid in acetic acid[8] for 4-h periods.

rhamnose. As indicated in Table II, the degree of 3-*O*-methylation is sensitive to changes in the composition of the growth medium.

Methylation analyses[6] (see Table III) of carboxyl-reduced CPS and EPS of strain 1B3 showed that the L-rhamnopyranose residues occur exclusively as (non-reducing) end groups; *i.e.*, permethylation also converted the 3-*O*-methyl-L-rhamnose

into the 2,3,4-tri-*O*-methyl derivative. The D-mannopyranosyl and two of the four D-glucopyranosyl units are unbranched, (1→3)-linked residues. Two types of branching occur: 1,3,6-tri-*O*-substituted D-glucopyranosyl residues, and 1,3,4-tri-*O*-substituted D-galactopyranosyl residues derived from D-galacturonic acid residues in the native polysaccharides.

Brief formolysis[7] of the methylated EPS readily liberated the mannose and half of the glucose derivatives, in addition to those of galactose and rhamnose. This result suggests that the resistant glucosyl residues occur as a β-(1→3)-linked, disaccharide (laminarabiose) unit. Formolysis is important for establishing the proportion of rhamnosyl groups because, as shown in Table III, more-extensive acid hydrolysis[8] degrades them.

From the methylation analysis, it was evident that the unmethylated L-rhamnosyl end-groups would be the only residues susceptible to oxidation with periodate. Periodate consumption, and liberation of formic acid, proceeded to overoxidation. Extrapolation of these curves back to zero time gave values of ~4.0 mol of periodate consumed and half that amount of formic acid liberated per decasaccharide repeating-unit of the carboxyl-reduced CPS. These values are those expected for the oxidation of the rhamnopyranosyl end-groups.

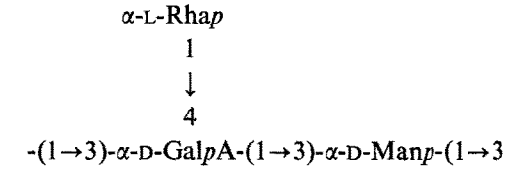
To confirm this result, the oxidized (80 h at 20°), carboxyl-reduced EPS was reduced with $NaBH_4$, and the product subjected to mild hydrolysis with 0.5M H_2SO_4 for 16 h at 20°. A portion of this Smith-degradation[9] product was further hydrolyzed, with 2M HCl for 1 h at 100°, and the hydrolyzate was analyzed by g.l.c. of the PAAN derivatives; only a trace of L-rhamnose remained (see Table II). A second portion of the Smith-degradation product was permethylated. The methylation-fragmentation products listed in Table III indicate a single, L-rhamnosyl (nonreducing) end-group and a corresponding 1,3,6-tri-*O*-substituted D-glucosyl branch-point per octasaccharide repeating-unit. A decrease in the proportion of 2,4-di-*O*-methylglucose and a corresponding increase in 2,4,6-tri-*O*-methyl-D-glucose indicated that another L-rhamnosyl group had been attached to O-6 of one of the glucose residues. Replacement of the 2,6-di-*O*-methyl PAAN derivative of D-galactose by a 2,4,6-trimethyl ether demonstrates that one of the L-rhamnopyranosyl groups had been appended to O-4 of the galactosyluronic acid residue. The CPS has more periodate-resistant 3-*O*-methyl-L-rhamnose than has the EPS, and methylation of its Smith-degradation product gives more tri-*O*-methylrhamnose and 2,4-di-*O*-methylglucose. The optical rotation of the Smith-degraded CPS before carboxyl-reduction, $[\alpha]_D^{20}$ +140° (*c* 1.43, water), in contrast to that of the native CPS, $[\alpha]_D^{20}$ +83° (*c* 0.97, water), indicates that the L-rhamnosyl end-groups are of the α-anomeric configuration.

Partial hydrolysis of the native CPS led to formation of aldobiouronic and aldotriouronic acids. The partial hydrolyzate was treated with an anion-exchange resin to adsorb the acidic components. These were eluted with 1.7M acetic acid, and the biouronic and triouronic acids were then separated on a column of Biogel P2. Acid hydrolysis of the aldobiouronic acid, $[\alpha]_D^{20}$ +66° (*c* 0.24, water), and visual examination of the hydrolyzate by thin-layer chromatography (t.l.c.) revealed the

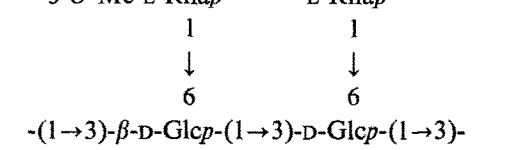
presence of D-mannose and D-galacturonic acid in equal amounts. The aldotriouronic acid contained D-glucose, in addition to these components. Prior to methylation analysis, the aldobiouronic acid was reduced with sodium borodeuteride, and the product converted into the methyl ester as described by Haaland[10]. Following reduction of the ester with $NaBD_4$, the product was permethylated, the ether hydrolyzed, and the sugars derivatized. The mixture of PAAN derivative and alditol acetate was analyzed by g.l.c.–m.s. Identification of 5-*O*-acetyl-6,6-dideuterio-2,3,4,6-tetra-*O*-methyl-D-galactononitrile and a 3-*O*-acetyl-1-deuterio-1,2,4,5,6-penta-*O*-methylhexitol in approximately equal amounts confirmed the structure of the aldobiouronic acid as 3-*O*-(α-D-galactopyranosyluronic acid)-D-mannose.

The aldotriouronic acid fraction was treated in the same way. Methylation analysis revealed (*a*) the presence of a minor trisaccharide, and (*b*) that some degradation had occurred during formation of the methyl ester, as indicated by the presence of a hexa-*O*-methylalditol. Hydrolysis products of the principal, permethylated trisaccharide were identified, by g.l.c.–m.s., as the PAAN derivatives of 2,3,4,6-tetra-*O*-methyl-6,6-dideuterio-D-galactose and 2,4,6-tri-*O*-methyl-D-mannose, in addition to a 3-*O*-acetyl-1-deuterio-1,2,4,5,6-penta-*O*-methylalditol presumably derived from the D-glucose component. This result suggests a trisaccharide structure consisting of the previously identified aldobiouronic acid linked through the D-mannopyranosyl residue to O-3 of D-glucose.

The results of methylation analyses of intact and Smith-degraded forms of the carboxyl-reduced EPS and CPS are qualitatively similar to those obtained for *R. japonicum* USDA 110 and USDA 138 exopolysaccharides[11]. In the permethylated *R. japonicum* polysaccharides[11a], D-glucosyl residues also occur exclusively as 2,4-di- and 2,4,6-trimethyl ethers, and the D-mannosyl and D-galactosyl residues (from the reduced uronic acid) as the 2,4,6-trimethyl ethers. The aldo-bio- and -trio-uronic acids, α-GalA-(1→3)-Man and GalA-(1→3)-Man-(1→3)-Glc, identified in the present work may have arisen from similar, main-chain sequences. The following, partial structure for polysaccharide 1B3, which includes the oligosaccharide sequences, is proposed.



An additional, perhaps overlapping, sequence is inferred from the slow release of di-*O*-methylated glucose during the acid hydrolysis of permethylated PS, namely,



wherein the positions of the rhamnosyl and 3-*O*-methylrhamnosyl groups may be the reverse. The α-anomeric configurations of the L-rhamnopyranosyl groups are inferred from the increased dextrorotation of the Smith-degradation product, and that of the D-galactopyranosyluronic acid, from the optical rotation of the aldobiouronic acid. The ease of liberation of the mannosyl residues suggests that they, also, possess the α-anomeric configuration.

EXPERIMENTAL

Isolation and purification of the polysaccharides. — Dr. Joe C. Burton of the Nitragin Company, Milwaukee, Wisconsin, kindly provided the *Rhizobium* strains of the 1B series, a number of which originated from the NiFTAL collection, Paia, Hawaii. Growth on YMS, isolation, and purification of CPS from strain 1B3 were described previously[1].

Both EPS and CPS were isolated from rotary-shaken (200 r.p.m., 8 d at 25°) cultures of strain 1B3. Owens medium C[3] (0.5 L in 2.8-L Fernbach flasks) was modified, for better pH control, by using a mixed carbon-source, D-mannitol (8 g) and K gluconate (2 g) per liter, and by doubling (to 2 g/L) the amount of casamino acids.

Cells and CPS were harvested by centrifugation at 20,000*g* for 15 min. The EPS was precipitated from the clear culture-liquor, which had been made 1% (w/v) in KCl, by addition of 95% ethanol (3 vol.). The stringy precipitate was dispersed in water, and the EPS was precipitated as before. The reprecipitated gum was redispersed in water, and the dispersion dialyzed, made neutral with dilute KOH solution, and lyophilized.

The centrifugate was a voluminous, loose gel, which did not mix well with either water or buffered saline. As described for isolation of the CPS produced on YMS medium[1], treatment with 0.1 M KOH was the only successful means of separating the cells from their capsules. Neither pyruvic acetal[12] nor *O*-acyl[13] groups could be detected prior to this treatment. After the cells had been removed by centrifugation, the CPS was directly precipitated by addition of 95% ethanol (3 vol.). This precipitate was more gelatinous than that of the EPS.

Neither treatment with Cetavlon[14] nor incubation with the exo-β-(1→3)-glucanase[15] of *Cladosporium resinae* NRRL 6437 or the endo-β-(1→3)-glucanase[16] of *Rhizopus arrhizus* 6431 changed the component ratios in either of the polysaccharides formed on the modified, synthetic medium. A mixture of such glucanases has been successfully used[16a] in order to remove β-(1→3)-linked D-glucans from rhizobial-polysaccharide preparations. Gel-permeation chromatography on a Waters E-High A column indicated $\overline{M}_w$ for both native and carboxyl-reduced EPS in excess of 10^7. Analytical ultracentrifugation also indicated the absence of a second biopolymer.

Component analyses. — To achieve complete reduction of carboxyl groups, the polysaccharides were twice reduced by the procedure of Taylor and Conrad[4]. An earlier report[1] described component analyses as PAAN derivatives. Component

TABLE IV

RELATIVE RETENTION TIMES[a] OF TRIMETHYLSILYL ETHERS OF METHYL GLYCOSIDES FROM THE EPS OF STRAIN 1B3

Methyl glycoside of	*Methanolysis products*	
	Native EPS	*Reduced methanolyzate*
3-*O*-Methyl-α-L-rhamnose	0.44	0.44
α-L-Rhamnose	0.62	0.62
Methyl D-galactofuranuronate (anomeric pair)	0.87, 0.93[b]	—
α-D-Mannose	0.94	0.94
D-Galactofuranose	—	0.96[b]
β-D-Mannose	0.98	0.98[b]
α-D-Galactopyranose	—	**1.00**
Methyl D-galactopyranuronate	1.04	—
β-D-Galactopyranose	—	1.04
α-D-Glucose	1.07	1.07
β-D-Glucose	1.11	1.11

[a]On OV-1, 115–220°, 2°/min, held at limit; 1.00 = methyl α-galactopyranoside (33.8 min). Derivatives identified by comparison to standards, and confirmed[18,19] by m.s. [b]Minor contribution to overlapping peaks, and therefore neglected in quantitation of sugars (see Table II).

ratios in the native polysaccharides were also determined as trimethylsilyl ether derivatives[17] of the methyl glycosides following methanolysis with 2% methanolic HCl in Teflon-sealed tubes for 2 h at 100° (bath). Relative retention-times in g.l.c. of these derivatives on an OV-1 column (nickel alloy, 2.44 m × 3.18 mm) are given in Table IV. A portion of the methanolyzate was made neutral with pyridine, and evaporated to dryness. Methyl esters were reduced with 0.8% aqueous $NaBH_4$ for 16 h at 4°, the excess of borohydride was decomposed with acetic acid, and boric acid was removed by repeated evaporation with methanol. Following methanolysis as before, and neutralization with Amberlite IR-45 anion-exchange resin, the filtrate was evaporated, and the dried methyl glycosides were converted into Me_3Si ethers. The relative retention-times of these products are also listed in Table IV.

D-Galacturonic acid was quantitated as D-galactose in the reduced heteropolysaccharides, because complete methanolysis of the native materials could not be achieved without some degradation.

In m.s., the per(trimethylsilyl) ether of the methyl 3-*O*-methyl-L-rhamnopyranoside gave fragment ions at *m/z* 73, 103, 131, 133, 146, 157, 171, 187, 191, 215, 217, 247, 261, 277, and 305. The corresponding methyl D-galactopyranuronate gave ions at *m/z* 73, 89, 103, 131, 133, 146, 159, 191, 199, 204, 217, 259, 273, 275, 301, 305, 317, 333, 363, 391, 407, and 423; the furanose form gave *m/z* 73, 89, 103, 131, 133, 146, 147, 159, 161, 187, 217, 227, 277, 288, 301, 319, 391, and 423. The mass spectra of the remaining sugars have been described[18,19].

Methylation analyses. — Freeze-dried samples of carboxyl-reduced polysaccharides were permethylated, and the ethers isolated as described[6,7]. Brief formolysis

of the methylated product with 91% formic acid for 2 h at 100° was usually followed by hydrolysis according to Stellner *et al.*[8]. To minimize degradation of rhamnosyl groups, the duration of the hydrolyses, initially with sulfuric acid in acetic acid, and subsequently, diluted with an equal volume of water, was limited to 4 h at 80° in sealed tubes. Removal of sulfate ion, conversion of the methyl ethers into per-*O*-acetylaldononitrile (PAAN) derivatives, and g.l.c.–m.s. were conducted as described[6]. The g.l.c. temperature-program previously used (in a butanediol succinate column)[6] was modified, in order to accommodate the more-volatile tri-*O*-methylrhamnose and penta-*O*-methylalditol derivatives: 150–210° at 2°/min, and then held at 210°, with a helium-carrier flow-rate of 25 mL/min.

Methylated PAAN derivatives were identified by m.s., and according to relative, g.l.c. retention-times given by standards. The tri-*O*-methyl derivative of L-rhamnose was obtained by permethylation of neohesperidin. Standard mixtures of methylated glucose, mannose, and galactose PAAN derivatives were prepared from the corresponding methyl α-glycosides by partial Purdie[20] methylation followed by hydrolysis with M HCl for 1 h at 100°.

Acidic oligosaccharides were reduced with sodium borodeuteride before being converted into methyl esters by boiling for 24 h in dry methanol (5 mL) under reflux in the presence of dry, Dowex 50W-X8 (H^+) ion-exchange resin (200–400 mesh), according to Haaland[10]. Reduction of the ester with sodium borodeuteride converted the D-galactopyranosyluronic acid residues into 6,6-dideuterio-D-galactopyranosyl residues. Following permethylation as before, the products were isolated by partitioning between chloroform (10 mL) and water (20 mL). The chloroform layer was concentrated to 1 mL, washed repeatedly with water until all of the Me_2SO had been removed, dried with 4A molecular sieves, and evaporated; the permethylated oligosaccharides were hydrolyzed for 4 h at 80° according to the method of Stellner *et al.*[8]. Removal of sulfate ion and conversion of the hydrolysis products into peracetylated alditol and PAAN derivatives were conducted as already described.

Periodate oxidations. — Sufficient sodium periodate was added to a solution of polysaccharide (500 mg) in water (500 mL) to provide a calculated, 50% excess of the oxidant. Consumption of periodate was monitored by use of Avigad's method[21], and liberation of formic acid by titration after decomposition of unreacted periodate with ethylene glycol. Oxidations, conducted in the dark at 20°, proceeded rapidly during the initial 20 h, and thereafter, at a much lower, but steady, rate. Extrapolation of the linear, overoxidation curve to zero time gave a value of 3.6 mol of periodate consumed per decasaccharide repeating-unit (calculated value, 4.0 mol). Also in accord with exclusive oxidation of L-rhamnosyl (nonreducing) end-groups was the liberation of 1.8 mol of formic acid per repeating unit. Smith degradations[9] were conducted on carboxyl-reduced EPS that was oxidized for 80 h, at which time, the periodate consumed reached the level necessary to decompose the susceptible sugar-residues. The reaction was stopped by addition of an excess of ethylene glycol, and the oxidized polysaccharide was isolated by dialysis. After reduction with sodium borohydride, decomposition of the excess of reductant with M HCl, dialysis, and

neutralization of the acid with 0.1M NaOH, the degradation was completed by hydrolysis for 16 h in 0.5M sulfuric acid at room temperature. Degraded polysaccharides were isolated by dialysis of neutralized hydrolyzates, freeze-dried, and methylated as before. The CPS was treated similarly, except that it was carboxyl-reduced[4] after periodate oxidation, reduction with $NaBH_4$, and mild hydrolysis.

Partial hydrolysis, and isolation, of acidic oligosaccharides. — Native CPS (1 g) was dissolved in 2M hydrochloric acid (100 mL), the solution heated for 1 h at 100°, and cooled, the chloride ions were removed with silver carbonate, and the acidic sugars were adsorbed on Dowex 1-X4 (acetate) ion-exchange resin (100–200 mesh). The resin was washed exhaustively with water, to remove neutral sugars, and analysis of the water wash by t.l.c. indicated the presence of 3-*O*-methylrhamnose, rhamnose, mannose, and glucose. The acidic oligosaccharides were eluted with 1.7M acetic acid. Following removal of the acetic acid by evaporation, the acidic oligosaccharides were dissolved in water, and analysis by t.l.c. indicated the presence of a biouronic and a triouronic acid; these were separated by gel-permeation chromatography on a column (2.5 × 30 cm) of Biogel P2 (-400 mesh) at a flow rate of 0.2 mL/min.

Optical rotations were measured in a Bendix automatic, electronic polarimeter.

ACKNOWLEDGMENTS

We thank Kenneth Sexson for technical assistance with analytical ultracentrifugation, Dr. William E. Dick, Jr., for advice and discussions, and Dr. Karl L. Smiley and Dwight E. Hensley for samples of endo- and exo-β-(1→3)-glucanases.

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