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STRUCTURAL INVESTIGATION OF THE ACIDIC POLYSACCHARIDE SECRETED BY *Zoogloea ramigera* 115

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ABSTRACT

Batch-culture growth of *Zoogloea ramigera* 115 in a defined medium produced a weakly acidic polysaccharide containing glucose and galactose residues, and (*S*)-pyruvic acetal groups. Analytical results indicated that the polysaccharide does not have a simple repeating-unit. Mainly with the aid of Smith degradation of the native polysaccharide and oxidation and subsequent β-elimination of the methylated and then depyruvylated polysaccharide, some structural features of the polysaccharide were identified.

INTRODUCTION

The acidic polysaccharide secreted by *Zoogloea ramigera* 115, first isolated by Friedman and Dugan[1], forms highly viscous solutions and has a strong affinity for several metal ions[2]. Some of the structural elements now reported for the polysaccharide are incompatible with the previously suggested, tentative structure[3]. The polysaccharide probably has an irregular structure with a composition that varies due to slight differences in the growth conditions of the organism, and hence a complete structural elucidation was not attempted.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at <40° (bath). G.l.c. was performed with a Perkin–Elmer 3920 or Sigma 1 gas chromatograph, each fitted with a flame-ionisation detector, and a glass column (200 × 0.3 cm) containing 3% of ECNSS-M on Gas Chrom Q for alditol acetates or an LKB SE-30 W.C.O.T., glass-capillary column (25 m × 0.25 mm) for partially methylated alditol acetates and partially methylated, partially ethylated alditol ace-

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tates. For g.l.c.–m.s. (e.i.), the appropriate g.l.c. column was used in a Finnigan 4021 quadrupole instrument. Mass spectra were recorded at an ionisation potential of 70 eV. A Perkin–Elmer 241 photoelectric polarimeter was used to determine optical rotations.

Isolation of the polysaccharide. — *Zoogloea ramigera* 115 (ATCC 25 935) was grown in a fermentor with a defined medium[4] containing D-glucose as the carbon source. Each batch culture was harvested after 120 h, and the polysaccharide was isolated according to the scheme devised by Jeanes *et al.*[5].

Sugar analyses. — Polysaccharide (2 mg) was treated with 0.25M H_2SO_4 (3 mL) for 18 h at 100°. The hydrolysate was neutralised with barium carbonate, filtered, and reduced with sodium borohydride. After acidification with glacial acetic acid, borate was removed by repeated co-concentration with methanol. The residue was treated with acetic anhydride–pyridine for 20 min at 100°, excess of anhydride was decomposed with ethanol, and the resulting solution was concentrated to dryness. After partition between water and chloroform, the alditol acetates obtained were analysed by g.l.c.–m.s.[6,7].

Alkylations and methylation analyses. — Methylations and ethylations were performed by the Hakomori method[8], and methylation analyses as previously described[9]. Because of limited solubility, dilute solutions in methyl sulphoxide were used when native polysaccharide was methylated.

Paper chromatography and colorimetric assays. — Analytical p.c. of polysaccharide hydrolysates (0.25M H_2SO_4, 18 h, 100°) was performed with ethyl acetate–acetic acid–water (3:1:1) and Whatman No. 1 paper, with detection by alkaline silver nitrate[10]. The polysaccharide was tested for uronic acids by the carbazole method[11]. Pyruvic acid was determined as the 2,4-dinitrophenylhydrazone[12]. After gel-permeation chromatography, the fractions obtained were analysed for hexoses by the anthrone method[13].

Investigation of the native polysaccharide. — *(a) Mild, acid hydrolysis.* The polysaccharide was treated with 0.1M HCl at 80° for 1.5 and 4 h. The solutions were then dialysed and lyophilised, and parts of the recovered materials were subjected to methylation analysis.

(b) Smith degradation[14]. A solution of the polysaccharide (100 mg) in 0.1M sodium acetate buffer (50 mL, pH 4.0) containing sodium metaperiodate (300 mg) was stirred in the dark at room temperature for 3 days. Excess of periodate was then reduced by stirring overnight with ethylene glycol (1 mL). The solution was dialysed and concentrated (to 40 mL), sodium borohydride (800 mg) was added, and the mixture was left at room temperature overnight. Excess of reductant was decomposed by the dropwise addition of acetic acid, and the solution was dialysed and lyophilised. A small part of the recovered material was subjected to sugar and methylation analyses. Half of the remaining material was hydrolysed in 0.1M HCl (40 mL) at 80° for 1 h. After dialysis, the solution was concentrated and applied to a column of Sephadex G-15, to yield a single sugar-containing peak (anthrone). The appropriate fractions were then combined and lyophilised. A small part of the

recovered material was subjected to methylation analysis. The remainder of the material was methylated, and treated with 50% acetic acid at 100° for 4 h, and the solution was concentrated to dryness. The residue was dissolved in chloroform, and two aliquots were each concentrated to dryness. One residue was methylated and the other was ethylated. Each sample was then hydrolysed, reduced, acetylated, and analysed by g.l.c.–m.s. The main part of the hydrolysis product was purified on a column of Sephadex LH-20.

Carboxyl-reduction and mild, acid hydrolysis of the methylated polysaccharide. — To a solution of the methylated polysaccharide (50 mg) in dry tetrahydrofuran (5 mL) was carefully added lithium aluminium hydride (100 mg), and the resulting suspension was heated for 2.5 h at 70° and then left overnight at room temperature. Water-saturated ethyl acetate was added dropwise and finally a little water. The solids were then collected and washed with chloroform, and the combined filtrate and washings were concentrated to dryness. The residue was treated with 50% acetic acid at 100° for 2 h, dialysed against tap water, and lyophilised. A small part of the recovered material was ethylated, hydrolysed, reduced, acetylated, and analysed by g.l.c.–m.s.

Oxidation and base-catalysed β-elimination. — The foregoing methylated and depyruvylated (reduction, weak hydrolysis) polysaccharide was subjected to two cycles[15] of oxidation, base-catalysed β-elimination, and mild hydrolysis with acid. After the first oxidation step, the product was dialysed against tap water. After the second oxidation step and after the steps involving mild hydrolysis with acid, the products were purified by elution from a column of Sephadex LH-20 with methanol–chloroform (1:1). Fractions containing (partially) methylated sugars were identified (anthrone) and combined. Part of the material recovered after each cycle was ethylated, hydrolysed, reduced, acetylated, and analysed by g.l.c.–m.s.

^{1}H-N.m.r. spectroscopy. — A Varian 200-MHz Fourier-transform n.m.r. spectrometer was used with 5-mm tubes and solutions (5 mg/mL) in $CDCl_3$ of methylated polysaccharide, methylated carboxyl-reduced depyruvylated polysaccharide, depyruvylated and methylated polysaccharide, and the partially methylated product derived from the periodate-oxidised polysaccharide. Spectra were recorded at room temperature with a pulse of 5 μs, a total acquisition time of 3 s, a sweep width of 2.6 kHz, and a memory of 15 k. Chemical shifts were assigned relative to that of internal $CHCl_3$ (δ 7.26). Periodate-oxidised polysaccharide that had been mildly hydrolysed was twice dissolved in D_2O (99.75%) and lyophilised. The spectrum of a solution (10 mg/mL) in D_2O (99.75%) was then recorded at 90°, using the above parameters. The HOD peak, which was diminished by a special pulse sequence[16], was well removed from the β-anomeric region at the temperature used. Chemical shifts were assigned relative to that of internal sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (δ 0.0).

RESULTS AND DISCUSSION

Sugar analysis of the polysaccharide revealed glucose and galactose in the ratio ~2:1, and neither p.c. nor g.l.c.–m.s. showed any other glycose residues. Elementary analyses for P and S were negative. No uronic acids were detected by the carbazole assay[11]. However, when a solution of the polysaccharide (free of external salts) was passed through a cation-exchange (H^+) resin, it became acidic (pH 3.2). As shown below, this was due to pyruvic groups.

Methylation-analysis data for the polysaccharide are shown in Table I. For two batches of the polysaccharide, there was considerable variation in the relative amounts of the two partially methylated alditol acetates eluted last (*cf.* 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylgalactitol), but it was more difficult to assess small differences in the amounts of the other partially methylated alditol acetates with any certainty. However, it is clear from the data that the polysaccharide cannot have a completely regular structure, since the proportions of the components are non-stoichiometric for a reasonable size of repeating unit. In all of the following analyses, polysaccharide from batch *A* was used, except for the colorimetric assay to determine pyruvic acid.

Methylation analysis showed that a little more than half of the originally 3,4,6-trisubstituted glucosyl residues had been replaced by 3-substituted glucosyl residues after mild, acid hydrolysis of the native polysaccharide for 1.5 h. After hydrolysis for 4 h, this conversion was virtually complete. Analysis of methylated polysaccharide that had been carboxyl-reduced, subjected to mild hydrolysis with acid, and ethylated showed that all of the 3,4,6-trisubstituted glucosyl residues in the original polysaccharide were still substituted (presumably glycosylated) at O-3, whereas O-4 and O-6 were ethylated. This finding pointed to a cyclic acetal, and a

TABLE I

METHYLATION ANALYSES OF NATIVE AND PERIODATE-DEGRADED POLYSACCHARIDE FROM *Zoogloea ramigera* 115

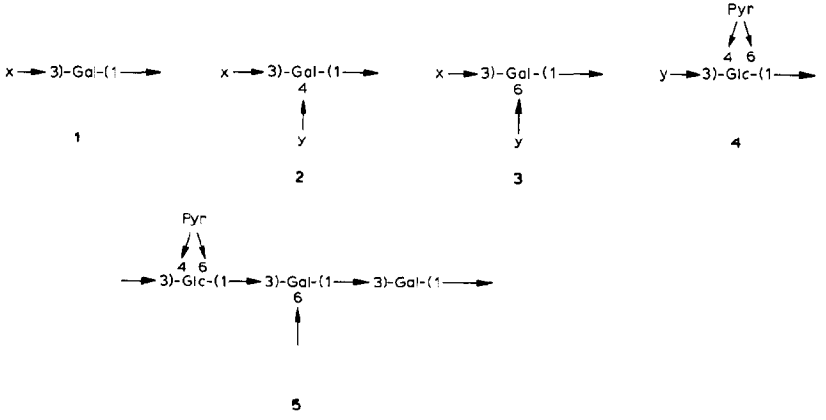
Glycosyl residue	*Position of O-methyl groups*	T[a]	*Sample*[b] *(mol %)*		
			A	B	C
Glc	2,3,4,6	1.00	22	23	—
Glc	2,3,6	1.42	30	31	2
Gal	2,4,6	1.45	13	15	27
Gal	2,6	1.73	13	16	28
Glc	2,3	2.06	1	1	2
Gal	2,4	2.33	10	6	20
Glc	2	2.61	11	7	20

[a]Relative retention times on the SE-30 column at 170°. The 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol obtained in the methylation analysis of depyruvylated polysaccharide had *T* 1.34. [b]Samples *A* and *B* were different batches of native polysaccharide. Sample *C* was periodate-oxidised and reduced ($NaBH_4$) polysaccharide *A*.

^{1}H-n.m.r. singlet at δ 1.48 for the methylated polysaccharide indicated the presence of a pyruvic acid acetal. The absolute configuration of the pyruvic acid acetal is *S*, since it appears 4,6-linked to glucose[17]. A colorimetric assay[12] indicated 3.6% of pyruvic acid, which accorded with the amount predicted from the corresponding methylation analysis (batch *B*).

Methylation analysis showed that the periodate oxidation of vicinal diols in the polysaccharide was essentially complete (Table I). Sugar analysis showed that the product contained glucose and galactose in the ratio ~1:3. After treatment of the degradation product with 0.1M HCl at 80° for 1 h, methylation analysis revealed 3-substituted galactose and small proportions of terminal and pyruvylated terminal (4,6-disubstituted) glucose. Treatment of the methylated degradation-product with 50% acetic acid at 100° for 4 h followed by methylation or ethylation gave mainly 3-substituted galactose and 4,6-disubstituted glucose. This finding confirmed that the only new termini appearing after mild hydrolysis originated from the pyruvylated glucosyl residues. It is also clear that, in the original polysaccharide, differently substituted galactosyl residues cannot be interspaced anywhere by 4-substituted glucosyl residues, but must be linked to each other. As expected, analysis following ethylation revealed four different components, three of them containing *O*-ethyl groups. The original 3,4,6-trisubstituted glucosyl residues had become ethylated at O-3 and the two branched galactosyl residues had become ethylated at O-4 and O-6. A trace of the original 3,4-disubstituted galactosyl residues remained intact. The foregoing evidence accords with the presence of the structural features **1–4** in the polysaccharide, where x is any glycosyl residue substituted at O-3, and y is a terminal or 4-substituted glucosyl group.

Analyses after the first oxidation–degradation sequence revealed the incorporation of *O*-ethyl groups at O-3 of the original 3,6-disubstituted galactosyl residues of the native polysaccharide. Only traces of the original 3- and 4-substituted glycosyl residues had become ethylated. A small proportion of unoxidised, depyruvylated glucosyl residues remained, and approximately the same proportion of



non-ethylated, original, 3,6-disubstituted galactosyl residues. There was also loss of terminal glucosyl groups, whereas the relative amounts of the other glycosyl residues remained intact. In the analytical procedures following the second oxidation–degradation sequence, some of the original 3-substituted galactosyl residues had become ethylated at O-3. The amount corresponded approximately to that of the original 3,6-disubstituted galactosyl residues released after the first oxidation–degradation. Depyruvylated glucosyl residues left unoxidised after the first oxidation were oxidised in the second oxidation. The results indicated that the same glycosyl residue was released as in the first case. Again, there was loss of terminal glucosyl groups, whereas the 4-substituted glucosyl and the 3,4-disubstituted galactosyl residues remained intact. The analyses after the two reaction sequences together define trisaccharide **5** as a structural element of the polysaccharide.

The results of the methylation analyses show that most of the glycosyl residues must be pyranoid. The observation that virtually no hydrolysis of glycosidic linkages was caused by 0.1M HCl at 80° for 4 h suggests that they are all pyranoid.

Although the signals were not well resolved, the ^{1}H-n.m.r. spectrum of the methylated polysaccharide clearly showed that most of the glycosidic linkages are β. However, a broad peak (δ 5.54) appeared in the α region. Repeated purification of the methylated polysaccharide on Sephadex LH-20 did not remove or diminish this peak. Broad peaks and poor resolution are consistent with the fact that the polysaccharide seems to have an irregular structure. Methylated and depyruvylated, as well as depyruvylated and methylated, polysaccharide still gave the signal(s) at δ 5.54, whereas there were changes in the β region (d, δ 4.7→4.5–4.6). Further evidence for the presence of at least one α linkage in the polysaccharide was provided by the periodate-degraded material. The ^{1}H-n.m.r. spectrum of non-methylated, periodate-degraded polysaccharide that had been mildly hydrolysed, twice subjected to D_2O exchange, and then dissolved in D_2O contained an unresolved peak at δ 5.21 in the α region. In the β region, there was a doublet at δ 4.76 ($J_{1,2}$ 7.2 Hz) and unresolved signals at δ 4.6–4.7. The $[\alpha]_D^{25}$ value [+45° (*c* 0.4, water)] indicated that, if all the sugars were D, not all of the glycosidic linkages could be β. Methylated, periodate-degraded polysaccharide that had been mildly hydrolysed and then purified on Sephadex LH-20 gave a ^{1}H-n.m.r. spectrum ($CDCl_3$) having one doublet in the α region and three doublets in the β region (Table II). Since depyruvylation of the polysaccharide only resulted in changes in the β region, one of the galactosyl residues should be α.

TABLE II

^{1}H-N.M R. DATA FOR THE ANOMERIC PROTONS OF THE PARTIALLY METHYLATED PRODUCT OBTAINED FROM THE POLYSACCHARIDE AFTER PERIODATE OXIDATION, REDUCTION, METHYLATION, AND MILD HYDROLYSIS

Chemical shift (δ)[a]	4.45	4.62	4.73	5.28
Observed $J_{1,2}$ (Hz)	7.6	7.7	7.2	3.6
Assigned anomeric configuration	β	β	β	α

[a]Relative to the signal for internal chloroform at δ 7.26.

The results of the present investigation do not accord with those of Ikeda *et al.*[3] in numerous respects. These authors reported 3-substituted glucosyl, but not 3-substituted galactosyl, residues to be present in the native polysaccharide, whereas our results imply the opposite. The glucose–galactose ratio was different from that found here. A possible structure was suggested which contained no α linkages, although it was noted that the value of the optical rotation of the polysaccharide was higher than those found for well-known β-D-glucans. It was suggested that terminal glucosyl groups were attached to O-3 of 3,4-disubstituted galactosyl residues, and 4-substituted glucosyl residues to O-3 of 3,6-disubstituted galactosyl residues. The results of the Smith degradation noted above preclude all such structural features. Furthermore, no glycosyl residues surviving periodate oxidation could be attached to O-6 of the 3,6-disubstituted galactosyl residues. We find that at least the main part of the internal pyruvylated glucosyl residues are linked to O-3 of 3,6-disubstituted galactosyl residues, whereas Ikeda *et al.*[3] proposed that they are linked to 3-substituted glucosyl residues. Our polysaccharide preparations contained a trace of terminal, pyruvylated glucose, whereas a higher proportion was found in the polysaccharide analysed by Ikeda *et al.*[3]. It is possible that some or all of these structural differences arise from differences in the strain or growth conditions of the micro-organism.

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