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Note

The role of *O*-acetyl groups in the biosynthesis of alginate by *Azotobacter vinelandii*

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Alginic acid is a universal constituent of brown algae (*Phaeophyta*)[1]. Its occurence is otherwise limited to such bacteria as *Azotobacter vinelandii*[2], and a few strains of *Pseudomonas*[3]. The exopolysaccharide produced by these bacteria is, like the algal product, a (1→4)-linked copolymer of β-D-mannuronic acid (M) and α-L-guluronic acid (G) containing M-blocks and G-blocks interspaced with heteropolymeric sequences characterised by a high transition frequency[4]. Both monomer composition and block structure may vary, depending upon the organism and growing conditions. The most conspicious difference between algal and bacterial alginate is the presence of various amounts of *O*-acetyl groups in the latter. Davidson *et al.*[5] found that, in an alginate from *Azotobacter vinelandii*, the *O*-acetyl groups were associated with the mannuronic acid-rich regions of the polymer, and suggested that their function was to protect the mannuronic acid residues from conversion into guluronic acid residues by mannuronan C-5-epimerase. This enzyme, which is active during the biosynthesis of alginate, is liberated by the bacteria into the culture medium[6].

We have investigated the protective role of the acetyl groups by treating bacterial alginate before and after deacetylation with a highly purified epimerase enzyme. The monomeric composition and sequential structure of the product were characterised by ^{1}H-n.m.r. spectroscopy. The acetylation pattern and the structure of the unmodified alginate were analysed by degradation with a specific poly-α-L-guluronate lyase and examination of oligomers from the digest by n.m.r. spectroscopy.

The C-5 epimerase from *A. vinelandii* can epimerise bacterial and algal alginate having a wide range in monomer composition and sequential structure[7]. When the enzyme acted upon a highly polymeric substrate, the highest degree of conversion was obtained with alginate having an extreme homopolymeric block-structure. In order to investigate the protective role of the *O*-acetyl groups, an alginate having a high content (22%) of acetyl and a low content of transition diads

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(F_{MG}, F_{GM} = 0.03) was chosen. A purified, active epimerase was incubated with both the native acetylated alginate and a deacetylated sample, and the products were analysed by ^{1}H-n.m.r. spectroscopy. From the results in Table I, it is evident that the presence of *O*-acetyl groups strongly inhibits epimerisation. In the deacetylated sample, the content of guluronic acid (F_G) had increased from 0.45 to 0.68 ±0.01, whereas in the native, acetylated polymer only a minor increase in "G"-content could be detected after the treatment with the enzyme. Since only 20% of the residues were acetylated, one acetyl group must be able to protect more than one mannuronic acid unit from epimerisation.

The distribution of *O*-acetyl groups in the native polymer was investigated by degradation with a specific poly-L-guluronate lyase; the resulting oligomers were separated by gel filtration and analysed by ^{1}H-n.m.r. spectroscopy. The lyase from *Klebsiella aeruginosa* cleaves the G–G and G–M bonds specifically[8] for the guluronate residue in the glycon position, leaving a reducing guluronic acid residue and an unsaturated uronic acid (Δ, originating either from G or M) on the non-reducing end. The elution pattern is shown in Fig. 1. As expected for the action of a specific G-lyase on an extreme homopolymeric block alginate, the digest gave one major peak near the void volume of the column, containing high-molecular-weight material (45%), and one large fraction containing low-molecular-weight material (27%). Intermediate were three small peaks containing oligosaccharides.

The 400-MHz ^{1}H-n.m.r. spectrum gave information on the uronic acid composition, the diad frequencies, the "G"-centered triads GGG, MGM, GGM, and MGG, and the content of *O*-acetyl. The molecular size (d.p.) was estimated on the basis of the resonances due to the end groups[9,10]; Fig. 2 shows the 400-MHz spectrum of Fraction III. The recovery after digestion and gel filtration was 75% and a slight, selective loss in mannuronic acid (8%) was probably due to the fact that the material eluted between fractions I and II was not collected. The results in Table II show that fraction I contained molecules with d.p. 40, composed of 95% of mannuronic acid. The few G-residues are predominantly situated at the reducing end or next to the non-reducing end, indicating that the native polymer contained long M-blocks. The void fraction was extensively acetylated (48%) and, on average, every second residue was acetylated. Fractions II–V had an increasing

TABLE I

COMPOSITION AND DIAD FREQUENCES OF BACTERIAL ALGINATE

Alginate	F_G	F_M	F_{GG}	F_{MM}	$F_{MG,GM}$	*Acetyl*
Native bacterial	0.45	0.55	0.42	0.52	0.03	22%
Epimerised native I[a]	0.51	0.49	0.50	0.48	0.01	22%
II	0.50	0.50	0.48	0.48	0.02	
Epimerised, deacetylated I	0.67	0.33	0.57	0.23	0.10	0
II	0.69	0.31	0.59	0.21	0.10	0

[a]I and II designate two different enzyme preparations

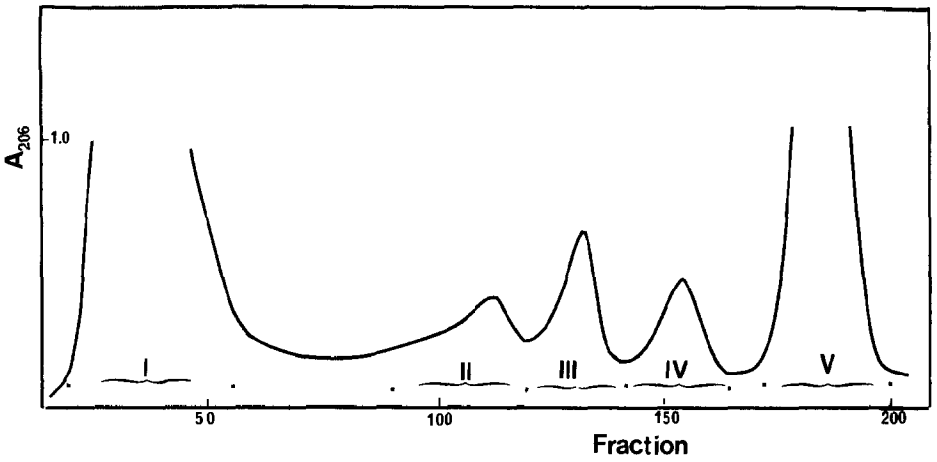

Fig. 1. Gel filtration of a poly-L-guluronate lyase digest of sodium alginate from *A. vinelandii* on Biogel P-4.

content of guluronic acid with decreasing chain length. Fraction II had a d.p. of 11 and contained 12% of M-units, more than half of which were involved in hetero-glycosidic linkages or next to the unsaturated end-groups. No acetyl could be detected in this fraction, indicating that the *O*-acetyl groups are associated only with the M-blocks. Fractions III–V had a d.p. of 8, 6, and 3, respectively, and contained >94% of guluronic acid but no *O*-acetyl.

Since the *Klebsiella* enzyme attacks oligosaccharides down to such pentamers as ΔGGGG and ΔMGGG, the presence of fractions II–IV indicated that the digestion had not proceeded to completion[8].

Thus, the acetyl groups in the bacterial alginate used in this work are exclusively associated with the M-blocks. On average, every second M-residues is acetylated, but the position and sequential arrangement of these groups are not known. The acetyl groups protect the M-blocks from attack by mannuronan C-5-epimerase, which is produced extracellularly by the bacteria. Since acetylation of alginate is an intracellular process, this provides the organism with a mechanism for controlling the degree of epimerisation of the exocellular polysaccharide and, hence, its physical properties such as ion-binding and gel-formation.

EXPERIMENTAL

Materials. — The *A. vinelandii* alginate was a gift from Dr. Sergio Paoletti (University of Trieste). It contained 45% of guluronic acid and 55% of mannuronic acid, had a degree of acetylation of 22%, and had a very low transition frequency (F_{MG}, F_{GM} = 0.03), indicating an extreme homopolymeric block-structure.

Enzymes. — The mannuronan C-5-epimerase was isolated from the liquid media of *A. vinelandii* by precipitation with ammonium sulphate, followed by

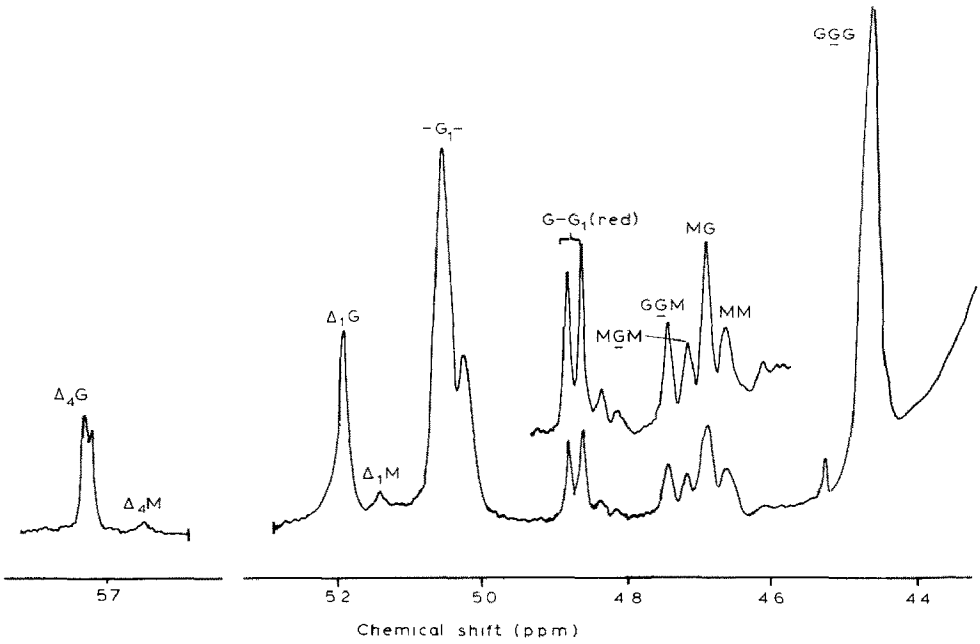

Fig 2. The anomeric region of the 400-MHz ^{1}H-n.m r. spectrum of fraction II from Fig. 1.

affinity chromatography on alginate–Sepharose[11]. The enzyme activity was assayed as the amount of tritium released into water when [5-^{3}H]alginate was incubated with the enzyme[12].

A specific poly-L-guluronate lyase was isolated from liquid cultures of the bacteria *Klebsiella aeruginosa* by precipitation with ammonium sulphate followed by ion-exchange chromatography on A-25 Sephadex[8]. The bacteria were kindly provided by Dr. J. R. Turvey (University College of North Wales).

Epimerisation. — To a solution of alginate (15 mg) in 0.05M collidine buffer (12 mL, 0.05M, pH 6.8) was added calcium chloride to 0.68mM, and the volume was adjusted to 19.5 mL with distilled water. The mixture was incubated with 0.5 mL of purified epimerase for 6 h at 30°. The reaction was stopped by adding 0.01M HCl to pH 3.5 and the samples for n.m.r. spectroscopy were prepared as described previously[13].

Degradation with poly-L-guluronate lyase. — To a solution of alginate (160 mg) in 25mM phosphate buffer (80 mL, pH 7.0) was added sodium chloride (0.8 g). The mixture was incubated with purified lyase (5 mg) at 30° for 24 h. U.v. absorbance at 230 nm was monitored. After 24 h, more (3 mg) enzyme was added, and the digestion was continued until there was no further increase in absorbance at 230 nm. The digest was concentrated to 10 mL, applied to a double column (2.5 × 100 mm) of Biogel P-4, and eluted with 0.1M sodium sulphate. Carbohydrate was detected by the u.v. absorbance at 206 nm. The oligouronides were desalted

TABLE II

COMPOSITION, DIAD, AND "G"-CENTERED TRIAD FREQUENCES OF OLIGOSACCHARIDES FROM A LYASE DIGEST OF BACTERIAL ALGINATE FROM *A. vinelandii*

Fraction	*Yield (mg)*	*O-Acetyl (%)*	F_G	F_M	F_{GG}	F_{MM}	F_{MG}	F_{GGG}	F_{MGM}	F_{GGM} F_{MGG}	$D.p._n$	*Reducing end*		*Non-reducing end*	
												F_G	F_M	$F_{\Delta G}$	$F_{\Delta M}$
Alginate	160	22	0.45	0.55	0.42	0.52	0.03				100				
I[a]	54.8	48	0.05	0.95							40	0.016	0.01	0.010	0.015
II	13.7	0	0.88	0.12	0.81	0.05	0.07	0.77	0.04	0.03	11	0.10	0.00	0.2	0.04
III	10.3	0	0.92	0 08							8				
IV	9.4	0	0.94	0.06							6				
V	34.2	0	0.96	0.04							3				

[a]See Fig. 1.

by gel filtration through G-15 Sephadex and freeze-dried. The low-molecular-weight material was desalted on charcoal.

^{1}H-N.m.r. spectroscopy. — The spectra were recorded with a Jeol FX-100 (98.8 MHz) or a Bruker VW (400 MHz) spectrometer. The monomer composition, diad and G-centered triad frequencies, the content of acetyl, and the d.p. were determined as described earlier[9,10,14]. The spectra of the acetylated samples were also recorded after deacetylation in order to obtain better resolution.

General methods. — Total carbohydrate was estimated by the phenol–sulphuric acid method, and the unsaturated uronic acid introduced by the lyase reaction was estimated by using the thiobarbituric acid reaction[15]. Deacetylation was carried out in 0.1M NaOH at 25° for 30 min.

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