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THE PURIFICATION AND CHEMICAL CHARACTERISATION OF THE ALGINATE PRESENT IN EXTRACELLULAR MATERIAL PRODUCED BY MUCOID STRAINS OF *Pseudomonas aeruginosa*

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ABSTRACT

A rapid ion-exchange method has been used to purify the alginate from the extracellular material of mucoid strains of *Pseudomonas aeruginosa* isolated from the lungs of cystic fibrosis patients. The structure has been investigated by chemical analysis, infrared spectroscopy, paper chromatography, and gas–liquid chromatography. The alginates contain mainly random or poly(D-mannuronic acid) block structures, and are highly acetylated. The relative viscosity is not correlated with the ratio of D-mannuronic acid to L-guluronic acid residues, or the degree of acetylation. The chemical/physical properties of the alginate from *P. aeruginosa* are considered in the context of the growth of the organism in the lung.

INTRODUCTION

Alginates are (1→4)-linked glycuronans comprised of residues of β-D-mannosyluronic acid (M) and α-L-gulosyluronic acid (G) arranged in block structures[1], which can be homopolymeric [poly(β-D-mannosyluronic acid) (MM) and poly(α-L-gulosyluronic acid) (GG)] or heteropolymeric [often occurring[2] as alternating blocks (MG)]. The gelling characteristics of the polymer in the presence of divalent cations, particularly[3] Ca^{2+}, depend on the M/G ratio, block structure, and extent of acetylation—*viz.*, the lower the M/G ratio, the stronger and more brittle is the gel that is formed[4], probably due to the formation[5] by G-blocks of "egg-box" structures, which strongly chelate Ca^{2+}.

Traditionally, alginates have been isolated from brown seaweeds and used commercially (*e.g.*, in the food industry) as gelling agents and stabilisers[6]. Two species of bacteria, *Azotobacter vinelandii*[7] and *Pseudomonas aeruginosa*[8], also produce alginate. The latter species is an opportunistic pathogen, commonly infecting burns and the respiratory tract[8]. Cystic fibrosis patients are particularly prone to infection with *P. aeruginosa*, and (unlike infection of burns) the organism usually assumes a mucoid phenotype which is characterised by the production of copious quantities of extracellular material. This material contains alginate, which has been identified previously on the basis of its chemical composition and i.r. spectrum[9].

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In view of the large effects that differences in alginate structure have on the gelling properties of the polymer, it is important to characterise the alginate from different clinical isolates if we are to begin to understand the significance of its production in cystic fibrosis patients. As part of a wider study of biochemical and clinical aspects of *P. aeruginosa* in cystic fibrosis, we have undertaken an investigation of the extracellular material isolated from clinical strains.

EXPERIMENTAL

Isolation and growth of organisms. — Primary isolation plates of sputum samples from cystic fibrosis patients were kindly provided by Mr. Alan Paull (Department of Medical Microbiology, Welsh National School of Medicine, Cardiff). Colonies of mucoid strains of *Pseudomonas aeruginosa* were selected and subcultured onto Pseudomonas isolation agar (PIA) (Difco) and incubated at 37° for 48 h.

General methods. — Protein and nucleic acid were quantified by measurement[10] of absorptions at 260 and 280 nm. For more accurate determination of protein, the method of Lowry *et al.*[11] was used with bovine serum albumin as a standard. Carbohydrate was assayed with the phenol–sulphuric acid reagent[12], and uronic acids were quantified with the carbazole–borate reagent[13], using D-mannurono-6,3-lactone as the standard. The *O*-acetyl content of the alginate samples was assayed by the method of Buscher *et al.*[14].

Extraction and purification of alginate. — Bacteria were removed gently from the surface of PIA plates by using a glass rod. The material obtained from three plates was combined, dispersed in water (500 mL), and centrifuged at 25,500*g* for 1 h at 4°. The supernatant solution, which contained the extracellular material, was removed carefully from the bacterial pellet, dialysed extensively against deionised water, and freeze-dried. These crude extracts were analysed for carbohydrate, protein, and nucleic acid and were subjected to i.r. spectroscopy.

Samples (~50 mg) of each crude extract were dissolved in the minimum of deionised water and applied separately to a column (9.5 × 1.5 cm) of Dowex 1-X2 (200–400 mesh; Cl^- form) resin equilibrated with 0.2M NaCl. Fractions were eluted from the column using a step-wise gradient of 100 mL each of 0.2–1.2M (in 0.2M increments) and 2, 3, 4, and 5M NaCl at a flow rate of ~5 mL.min^{-1}. The eluants from each step of the gradient were assayed for carbohydrate, uronic acid, and protein. Fractions containing uronic acids (see Results and Discussion) were combined, dialysed extensively against deionised water, and freeze-dried prior to further analysis.

^{1}H-N.m.r. spectroscopy. — The alginate samples were partially degraded by mild, acid hydrolysis (30 min, 100°, pH 2.9) in order to reduce the viscosity of the solutions, which were then neutralised and freeze-dried. The samples (5 mg) and EDTA (3 mg) were dissolved in D_2O (0.5 mL) at pD 7, and 360-MHz spectra were obtained at 90° with a Bruker WM-360 n.m.r. spectrometer operating in the

Fourier-transform mode. A 180°–τ–90° pulse sequence (τ ~3 s), with a re-cycle time of 5 s, was used to partially eliminate the solvent peak (HDO). ^{1}H-Chemical shifts were expressed in p.p.m. downfield from internal sodium 4,4-dimethyl-4-silapentanesulphonate. The assignment of the peaks was based on the work of Grasdalen *et al.*[15] and was confirmed by the use of algal-alginate block structures of known composition.

Infrared spectroscopy. — Dry samples of alginate were dispersed thoroughly in anhydrous KBr (1%, w/w) and pressed into discs, and the spectra recorded with a Perkin–Elmer 257 split-beam spectrophotometer.

Chromatography. — Samples of alginate were hydrolysed in aqueous 90% (v/v) formic acid for 6 h at 100°. The resulting uronic acids were separated by descending chromatography on Whatman No. 1 paper with butyl acetate–acetic acid–ethanol–water (3:2:1:1) and detected with alkaline silver nitrate[16].

One sample of alginate was also analysed by g.l.c., using the method of Clamp and Scott[17].

Viscosity measurements. — These were performed at 32.0° with an Ubbelohde viscometer having a nominal constant of 0.03 cS.s^{-1}. The viscosities of alginate solutions (0.75 mg.mL^{-1} of 0.1M NaCl) were measured (average of 10 readings) relative to that of the solvent.

RESULTS AND DISCUSSION

All the strains of *Pseudomonas aeruginosa* grew well on PIA plates, producing copious quantities of mucoid material. Invariably, the crude extracts of this material were found to be essentially free of bacterial contamination, as judged by the lack of detectable amounts of nucleic acids. Previously, it has been tacitly assumed that the water-soluble material extracted from the mucoid material consisted entirely of alginate that was partially acetylated[18]. However, the results in Table I show that both protein and non-uronic acid-containing polysaccharides are also present. The i.r. spectra of the crude material indicated the presence of amide groups (Fig. 1*a*), confirming the likely presence of a significant quantity of protein.

It was not possible to obtain pure preparations of alginate by varying either the conditions of the extraction from the culture media or by including several different washings (*e.g.*, with EDTA or NaCl) or precipitation (with propan-2-ol) steps in the isolation protocol. Therefore, in order to study the chemical and physical properties of alginate from clinical strains of *P. aeruginosa*, it was necessary to develop a convenient, routine method for its purification from the crude extracellular material. To this end, ion-exchange separation on Dowex 1-X2 was used. Typically, the protein and the bulk of the non-uronic acid-containing polysaccharide was eluted with 0.2M NaCl. The uronic acid-containing material was eluted with either 0.6 or 0.8M NaCl (Fig. 2*a*) to give an alginate-containing fraction. Occasionally, a small amount of uronic acid-containing material was found in the 1.0 or 1.2M NaCl eluates. Authentic algal-alginate samples were eluted from the Dowex 1-X2 column with 0.6 and 0.8M NaCl (Fig. 2*b*).

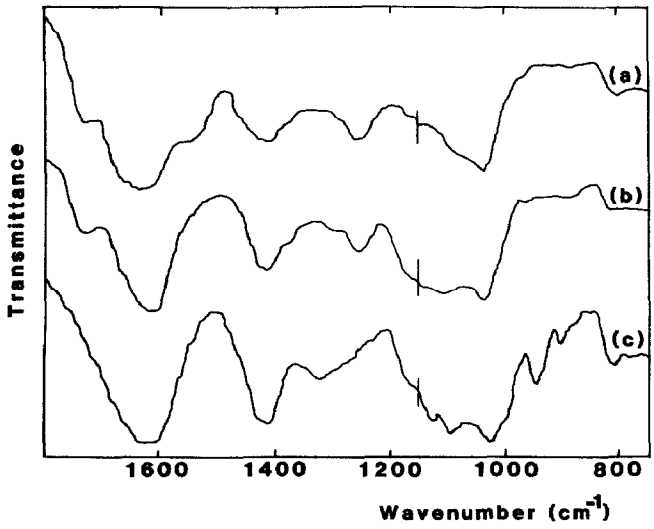

Fig. 1. Infrared spectra of (*a*) crude, cell-free extract from *P. aeruginosa* (strain 4335), (*b*) purified, cell-free extract (strain 4335), and (*c*) algal alginate (Manucol DH). Absorbance bands at 1250 and 1730 cm^{-1} are typical of the *O*-acetyl groups present in bacterial alginate.

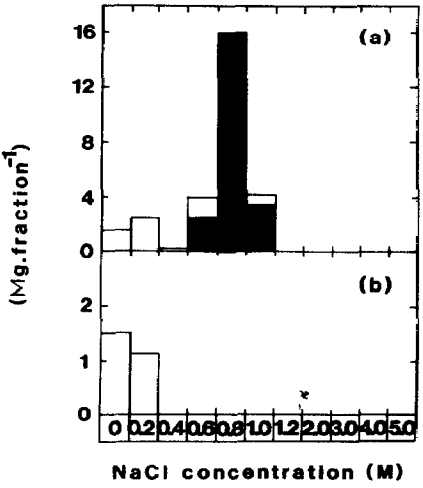

Fig. 2. Ion-exchange chromatography of the extracellular material from *P. aeruginosa* (strain 0096): (*a*) total carbohydrate (phenol–sulphuric acid assay) is indicated by the open blocks, and carbazole-positive material is indicated by the solid blocks; (*b*) protein content, measured by the Folin assay.

The alginate samples (combined 0.6 and 0.8M NaCl eluates) were assayed for uronic acid, total carbohydrate, and protein content. Typically, protein could not be detected and virtually all of the carbohydrate could be accounted for as uronic acid. Paper chromatography of formic acid hydrolysates of the alginate samples failed to reveal components other than those expected from an alginate-like

TABLE I

PERCENTAGE COMPOSITION[a] OF CRUDE WATER-SOLUBLE EXTRACTS OF *P. aeruginosa*

Sample	*Carbohydrate*	*Uronic acid*	*Protein*
7293	30.4	17.4	21.5
CF492a	60.8	34.0	20.0
0096	63.0	39.0	10.0
4335	42.8	20.0	21.5
3763	47.3	24.0	24.5
0046	25.0	20.0	14.0
2223	46.0	25.0	18.0

[a]The results are expressed as a percentage of the weighed, freeze-dried material. No attempt was made to account for the water content of the extracts. Neither nucleic acids nor 2-keto-3-deoxyoctulosonic acid (KDO) could be detected in any extract.

polymer. Semi-quantitative analysis of one of the samples by g.l.c. showed that D-mannuronic acid and L-guluronic acid were the major components. Small quantities (<1% of the total) of other sugars could be detected, but these were not considered to be significant. Analysis of the samples by i.r. spectroscopy produced typical alginate spectra, with no evidence of the amide bands that were present in spectra of the crude extracts (Fig. 1*b*).

Purified alginate samples obtained by chromatography on Dowex 1-X2 were analysed by ^{1}H-n.m.r. spectroscopy to establish the M/G ratio and to ascertain the extent and the type of block structures within the polymer. The ^{1}H-n.m.r. spectra were typical of alginates (Fig. 3), and the calculated doublet frequencies and fractional composition are shown in Table II. The range (1.27–3.55) of M/G ratios of the different samples obtained from *P. aeruginosa* is much narrower than that seen with alginates obtained from either brown seaweeds[19] or from the bacterium *Azotobacter vinelandii*[20]. Furthermore, there is no evidence of any G-block structure within the alginate isolated from *P. aeruginosa*. This finding indicates that these samples of alginate cannot form the "egg-box" structures, characteristic of strong inelastic gels, described by Rees[5].

Analysis of the alginate samples for their *O*-acetyl group content showed that all of the samples were esterified (Table III). Previous work[21] has suggested that the *O*-acetyl groups are associated exclusively with the D-mannuronic acid residues within the polymer. Our results indicate that most of the samples isolated from *P. aeruginosa* contain sufficient D-mannuronate residues to account for the extent of acetylation on a mole-for-mole basis. However, in several of the samples, the number of moles of *O*-acetyl groups exceeded the moles of available D-mannuronate. These data indicate that either a proportion of the L-guluronate residues can be esterified, or that some of the D-mannuronate residues can be *O*-acetylated at more than one hydroxyl group. The latter option is feasible, because there is no steric hindrance to substitution of β-D-mannuronate ($^4C_1$) by *O*-acetyl groups on both positions 2 (axial) and 3 (equatorial)[22].

TABLE II

THE FRACTIONAL COMPOSITION OF ALGINATES DERIVED FROM *P aeruginosa*

Sample	*Chemical composition*		*Doublet frequency*			
	F_G	F_M	F_{GG}	F_{MM}	F_{MG}	F_{GM}
7293	0.36	0.64	0	0.28	0.36	0.36
CF492a	0.26	0.74	0	0.48	0.26	0.26
0096	0.22	0.78	0	0.56	0.22	0.22
4335	0.24	0.76	0	0.52	0 24	0.24
3763	0.44	0.56	0	0.12	0 44	0.44
0046	0.26	0.74	0	0 48	0.26	0.26
2223	0.42	0.58	0	0.16	0.42	0.42

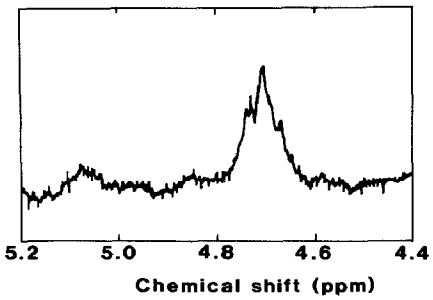

Fig. 3. N.m.r. spectrum of the purified alginate from *P. aeruginosa* (strain CF492a). The major peak at 4.65 p.p.m and the minor peak at 5.05 p.p.m. are indicative of a preparation consisting of MM and MG blocks. The absence of a peak at 4.45 p.p.m. indicates the lack of GG blocks in this sample.

Comparison of our data with those of other studies of the alginate from *P. aeruginosa* is complicated by the fact that different methods of isolation have been used, and that other workers have not used the ion-exchange (or any other similar) purification step. However, some other studies have found similar high M/G ratios[9,23,24], although the block structures were not delineated. McArthur and Ceri[23], using a g.l.c. method to analyse uronic acids, found 84% of D-mannuronate in the alginate of strain CF492a; we found 74% in the same strain using our methodology. Our acetylation values are higher than those of McArthur and Ceri[23], and Evans and Linker[9].

Measurement of the relative viscosities of the alginate samples derived from strains of *P. aeruginosa* revealed large differences in values (Table III). There is no direct correlation between the relative viscosity and either the M/G ratio or the degree of acetylation. It is most probable that the differences in values for the relative viscosity are related to differences in the molecular weight of the polysaccharides. Unfortunately, insufficient material was available to enable the intrinsic viscosity, and thus the molecular weight, to be determined.

In conclusion, it has been shown that the samples of alginate obtained from

TABLE III

THE *O*-ACETYL CONTENT AND RELATIVE VISCOSITY OF ALGINATE SAMPLES

Sample	*M/G ratio*	*Acetate/UA*[a] *ratio*	*Acetate/ManA ratio*	η_{rel}
7293	1.78	0.74	1.16	1.18
CF492a	2.85	0.62	0.84	1.21
0096	3.55	0.67	0.86	1.33
4335	3.17	0.58	0.76	1.10
3763	1.27	0.82	1.46	1.14
0046	2.85	0.70	0.95	1.08
2223	1.38	0.61	1.05	1.18

[a]Uronic acid.

isolates of *P. aeruginosa* contain mainly random or poly-M block structures, and are highly acetylated. The relative viscosity is not correlated with the degree of acetylation, and the lack of poly-G blocks probably means that these alginates do not gel with Ca^{2+} by forming "egg-box" structures. This finding is significant in the context of cystic fibrosis, because the Ca^{2+} concentration is elevated in the lung[25].

The formation of rather more elastic M-block-dependent gels may be an advantage in the natural environment of the producing micro-organism, *i.e.*, in the alveolar spaces of the lung, where there are large volume and shape changes associated with breathing. In addition, the bacteria grow as microcolonies inside lungs[26], and an elastic gel structure would enable the bacteria, which are motile, to grow, move, and divide within their protective alginate microenvironment.

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