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An enzymatic method for preparation of homopolymannuronate blocks and strictly alternating sequences of mannuronic and guluronic units

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Abstract

Two *Pseudomonas aeruginosa* alginates were lysed by an overexpressed polymannuronate lyase $AlxM_B$ (only acting on two or more consecutive, nonacetylated mannuronate units) to prepare either mannuronate blocks (poly-M blocks) with dp∼30, or strictly alternating sequences of mannuronic and guluronic acid (poly-MG blocks) with dp > 20. The poly-M blocks were obtained by lysis of a *P. aeruginosa* polymannuronate that has 50% O-acetylation at C-2 and C-3. The poly-MG blocks were obtained from a *P. aeruginosa* alginate that contained both mannuronate and guluronate residues. The polysaccharide was first deacetylated and then treated with the lyase to excise the mannuronate units from the alternating-MG blocks. Both types of blocks should have potent biological effects and should provide useful specific substrates for characterisation of other alginate lyases.

Keywords: Bacterial alginate; *Pseudomonas aeruginosa*; Alginate lyase; Mannuronate lyase; Poly-M-and poly-MG blocks

1. Introduction

Recently, alginate lyases have attracted attention because of their potential for expanding the uses of alginates [1–4]. These enzymes produce 4-deoxy-L-*erythro*-hex-4-ene pyranosyluronate-containing oligosaccharides which are thought to be promising biologically active substances [2]. Like oligosaccharines derived from fungi and plants [5,6], alginate-derived oligosaccharides were found to elicit germination-, shoot elongation- and root growth-promoting activities [7–9]. They also stimulate the growth of Bifidobacteria [10]. Fractions rich in β (1→4)-linked mannuronic acid have potent biological effects in several biological systems, which is probably due to a stimulation of the

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monocytes/macrophages [11–13]. Alginate lyases may also be used to characterise the fine structures of alginates and to correlate them with their solution properties [14]. They may also find use as therapeutic agents in the treatment of infections by mucoid strains of *Pseudomonas aeruginosa* [2,15].

Alginate lyases have been isolated from various sources [1–4] but the definition of their specificities is frequently a problem. Their substrate is a copolymer with a non-regular distribution of mannuronic(M) and guluronic(G) acid units [16,17], whereas the random distribution of *O*-acetyl groups on the 2 and 3 positions of mannuronate residues in bacterial alginates provides further complexity [18]. The enzyme specificity was usually determined by monitoring the degradation of whole alginates with high MM, or GG contents or MM and GG blocks prepared by partial acid hydrolysis [19]. These materials may, however, still contain significant proportions of heteropolymeric sequences, and lead to a misinterpretation of the enzyme specificity.

A rational use of alginate lyases in the evaluation of algae needs a perfect control of the activity and specificity profiles of these enzymes, and requires substrates of the highest possible purity. Taking advantage of the structure diversity of *Pseudomonas* alginates an enzymic method, utilizing a mannuronate lyase, was developed to prepare pure polymannuronic blocks (MM) with a degree of polymerization (dp) of about 30, and strictly alternating MG sequences, with dp > 20. Among the several mannuronate lyases described [2–4], an extracellular alginase from the marine bacterium ATCC 433367 seemed to be of interest [20,21]. This enzyme catalyzes depolymerization of the (1→4)-β-D-mannuronan block region in alginate with accumulation of uronides of various lengths (2–6 units) incorporating 4,5-unsaturated non-reducing terminal residues. From the structural analysis of the oligomers, it was suggested that the cleavage reaction required the interaction of the enzyme with at least three sequential mannuronic acid residues [22]. The recombinant plasmid pAL- A_3 bears this *endo*-mannuronan alginate lyase-encoding gene (alXM) [23]. The enzyme ($AlXM_A$) produced by *Escherichia coli* TC4/pAL-A3 was purified and the corresponding gene sequenced [24]. Overexpression of the enzyme in a slightly modified form, $AlXM_B$, has been achieved in *E. coli* BL21(DE_3)/pAL-Sur/pLys S [25]. $AlXM_B$ and $AlXM_A$ have identical alginate-degrading activities on various substrates including *P. aeruginosa* alginate. $AlXM_B$ was used throughout this work.

2. Experimental

Bacterial strains.—Two mucoid strains (8 and 12) of *P. aeruginosa* have been isolated from the sputa of cystic fibrosis patients and kindly provided by Dr. J.P Flandrois (Hôpital Jules Courmont, Lyon, France).

Growth conditions and isolation of bacterial alginates.—An 18 h preculture in Luria–Bertani medium was inoculated on PIA solid medium (*Pseudomonas* Isolation Agar, Difco) in Petri dishes (Ø = 10 cm) and incubated for 48 h at 37 °C. The bacterial culture was collected with a glass scraper and stirred in water. The cells were collected by centrifugation at 20,000 *g* for 20 min. Sodium chloride was added, until 1 M concentration, to the resulting supernatant and the solution was precipitated with 95% EtOH (v/v) at room temperature. The precipitate was successively washed with 60–95% EtOH and dried under vacuum. The yield was 64 mg and 90 mg per Petri dish for A-8 and A-12 alginate, respectively.

Methods.—Deacetylation of A-8 alginate and A-12 oligoalginate samples was carried out in NaOH (pH 11) at 80 °C for 1 h. After neutralisation, the polymer from A-8 was precipitated and collected according the method described above. The solution of deacetylated A-12 oligoalginates was ultrafiltred on a YC05 membrane (Amicon) and freeze-dried.

Chromatographic methods for the separation of the oligo-uronides have been described [26].

The M/G ratio and acetylation degree were determined from the 500 MHz ^{1}H NMR spectra by comparing the intensities of appropriate protons. Spectra were recorded using a Varian Unity Plus spectrometer with a 5 mm ^{1}H-^{13}C dual probe and assigned according previous results [27] and literature data [16–18].

A set of three blocks with different compositions, MM, GG and MG, respectively, were prepared by two cycles of HCl-hydrolysis of appropriate commercial marine alginate samples, according to the procedure of Haug et al. [19].

Enzyme and enzymatic assays.—Purified $AlxM_B$ alginate lyase was isolated from the recombinant *E. coli* BL21(DE3)/pLys S/pAl-Sur, as described by Malissard et al. [25]. Enzyme activity (85U/mg

protein) was measured using the *Macrocystis pyrifera* alginate and the thiobarbituric acid assay as described [25]. One enzyme unit produced 1 μmol of non-reducing, unsaturated termini per min.

The enzymic degradations of deacetylated A-8 and acetylated A-12 alginates were performed in 75 mM sodium phosphates, 450 mM NaCl buffer (pH 7.5). At 100 mL of a 0.2 % sodium alginate solution, 1 mL of enzyme preparation (110 U) was added and incubated for 7 h at 37 °C. The enzyme reaction was stopped by heating at 100 °C for 10 min and filtered. The degraded deacetylated A-8 sample was concentrated and directly loaded on a Bio-Gel P-6 column (100×2.5 cm). About 70% (125 mg) of the starting alginate (200 mg) were collected and identified with poly-MG blocks. The degraded A-12 sample was first deacetylated before fractionation and 50% (100 mg) of the starting acetylated alginate (200 mg) were recovered as poly-M blocks.

Molecular weight determination.—Alginates were characterised at room temperature by size exclusion chromatography (SEC) using a modified Waters 150C apparatus with multidetection: a differential refractometer for concentration, a capillary viscometer for intrinsic viscosity [η] determination and a multiangle laser light-scattering detector (MALLS) (DAWN F from Wyatt Technology) for molecular weight determination. Two gel filtration columns (8×300 mm) in series, Shodex OHpak SB-804 HQ and SB-805 HQ (from Interchim, France), were eluted at 1 mL/min with 0.1 M $NaNO_3$. The injected volumes were 200 μL (polymer concentration: 1 g/L).

Weight-average molecular weights of 2250 and 1550 KDa were determined for A-8 and A-12 alginates, respectively.

3. Results and discussion

Structural determination of two bacterial alginates.—*Pseudomonas aeruginosa* alginates contain various arrangements of M and G residues ranging from homopolymannuronic structures to strictly alternating MG sequence polymers. Like some other bacterial alginates, they are devoid of two or more consecutive G residues [18]. Unlike alginates from brown algae, bacterial alginates are *O*-acetylated at positions 2,3 (or both 2 and 3) of some of the D-mannuronic acid residue. The degree of acetylation depends upon the strain [18].

^{1}H NMR spectra of the two native alginates (A-8 and A-12) isolated from the corresponding mucoid *P. aeruginosa* strains are shown in Fig. 1. Alginate A-12 is consistent with a homopolymannuronate structure, whereas alginate A-8 contains both mannuronic and guluronic acid residues. Both polymers are *O*-acetylated.

With the help of published data [18], the spectra of the acetylated samples (Fig. 1A and B) were interpreted in terms of acetylation pattern. Both spectra display partly overlapping peaks in the 2 ppm region suggesting the presence of both mono- and di-acetylated units. The resonances between 5.05 and 5.30 ppm were assigned to the H-3 signals with O-3 acetylated, the signal at 5.05–5.15 ppm being due to H-3 with both O-2 and O-3 acetylated. The resonances for H-2 with O-2 acetylated were located at 4.8–4.9 ppm, overlapping with H-1 signals of guluronate units in the spectrum of the A-8 sample (Fig. 1A). By the direct addition of a drop of concentrated NaOD in the NMR tube, an almost instantaneous deacetylation was obtained (Fig. 2A and B) allowing facile determination of the M/G ratio and the acetyl group content, by integration of the characteristic protons. Composition and acetylation pattern are reported in Table 1.

Enzymatic preparation of poly-M blocks.—Solutions of high molecular-weight bacterial alginates are too viscous to work on. Therefore, an initial partial degradation of the native alginate was needed to prepare polymannuronate blocks with high dp. As acid-hydrolysis is tedious and hard to control on such viscous solutions, the use of a

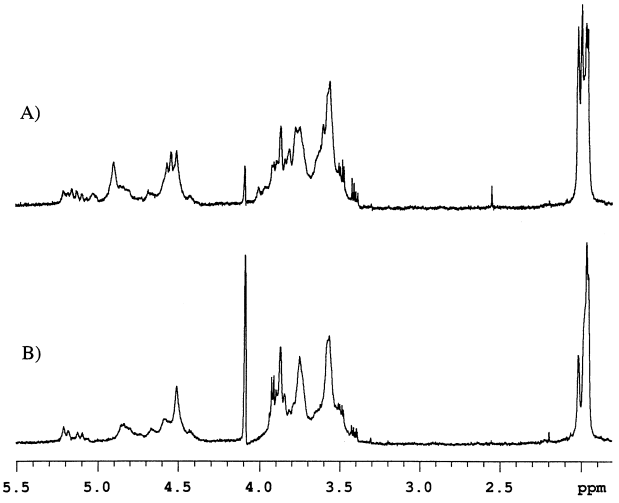

Fig. 1. ^{1}H NMR spectra of A-8 (1-A) and A-12 (1-B) samples at 80 °C from *Pseudomonas aeruginosa* alginates.

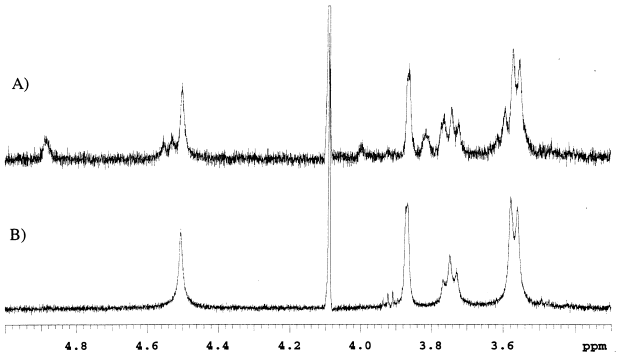

Fig. 2. ^{1}H NMR spectra of A-8 (2-A) and A-12 (2-B) samples at 80 °C after NaOD treatment.

mannuronate lyase was preferable, provided that the extent of hydrolysis could be limited so as to prevent the ultimate accumulation of oligomers with low dp ($2 \leq dp \geq 6$) [1,22,26]. Taking advantage of the more-limited activity of such an enzyme on *O*-acetylated alginate, the activity of $AlXM_B$ was tested on the native and deacetylated A-12 bacterial alginates and found to be 19 and 116 U/mg, respectively. Assuming that $AlXM_B$ is efficient only when at least two consecutive unsubstituted mannuronic acid residues are present, and knowing that there are more than 50% of *O*-acetyl groups and only 6–7% of diacetylated units (Table 1), the likelihood of achieving a limited breakdown of A-12 alginate by $AlXM_B$ with the concomitant production of large blocks, was good.

After incubation of A-12 alginate with $AlXM_B$, as described in the Experimental section, deacetylation was effected with sodium hydroxide. After ultrafiltration, the mixture was resolved by chromatography on a Bio-Gel P-6 column with 0.1 M sodium nitrate as eluent. The profile generated is shown in Fig. 3. Four fractions (I, II, III and IV respectively) were collected, desalted and analysed by ^{1}H NMR. Fraction IV (13 % w/w from deacetylated A-12) was a pure unsaturated trimannuronate; fractions II (24 %) and III (13 %) were

Table 1
Composition of A-8 and A-12 *Pseudomonas aeruginosa* alginates

Alginate	M/G	% Acet. (total)[a]	% 2-O-Acet.	% 3-O-Acet.
A-8	2.5	55-60	25–28	30–32
A-12	Poly-M	50–55	32–35[b]	18–20[b]

[a]An acetyl content of 100% points a polymer with one acetyl group on each monomer unit.
[b]With 6–7% of disubstituted mannuronic acid units.

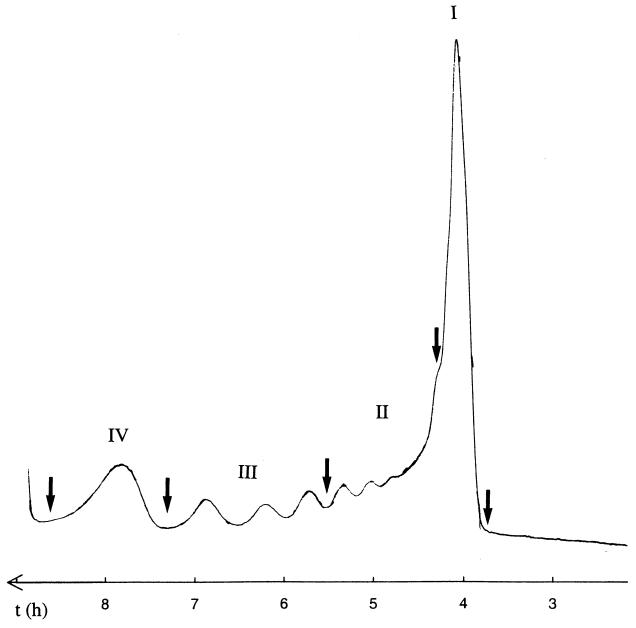

Fig. 3. Gel filtration on Bio-Gel P-6 of the deacetylated $AlxM_B$-digest of A-12 *Pseudomonas aeruginosa* alginate.

mixtures of unsaturated oligomannuronates with an average dp of 17 and 5.5, respectively (data not shown). Fraction I (50%) was obviously a polymannuronate block with $dp \simeq 30$ (poly-M) (Fig. 4).

Enzymic preparation of mannuronate blocks has been described previously [28]. The use of a guluronate lyase on a marine alginate resulted in the isolation of short blocks ($dp \simeq 15$–20) with a G content around 10%. This was similar to samples prepared by acid hydrolysis. Now, by combining the use of a mannuronate lyase with little or no activity against substituted mannuronic acid residues and a highly acetylated mannuronan from the bacterial alginate A-12, it was easy to prepare poly-M blocks that are virtually free from G.

Enzymatic preparation of poly-MG blocks.—To understand the rules governing the specificity of a given lyase and to rationalise the evaluation of alginates, studies of the enzyme's activity on heteropolymeric zones is needed. Long and regular MG-blocks, as substrates, cannot be isolated from marine alginates by acid or enzymic treatment, because of the limited range of sequences where mannuronic and guluronic units really alternate. Considering the structure of alginate A-8 from *Pseudomonas* strain (Fig. 2A), the 57% of uronic acid residues involved in strictly alternating MG sequences could supply the desired substrate after degradation of the homo-polymannuronate zones. $AlxM_B$ alginate lyase, active only against

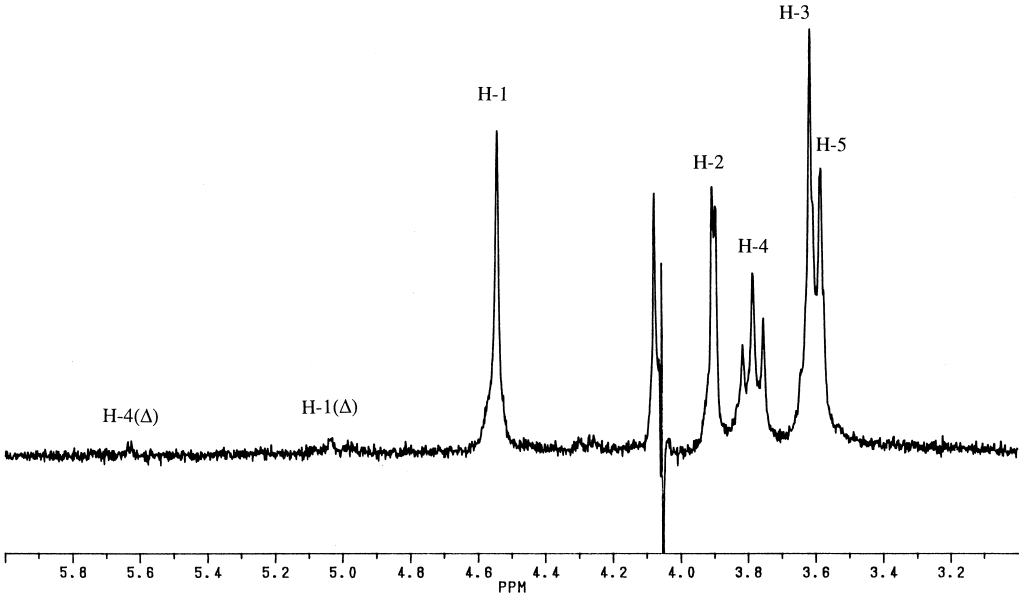

Fig. 4. ^{1}H NMR spectra at 85 °C of Fraction I from Fig. 3 (Δ refers to a 4-deoxy-L-*erythro*-hex-4-enopyranosyluronate unit).

poly-M blocks [29], was incubated, as described above, with 200 mg of deacetylated alginate A-8. Size-fractionation of the reaction products was performed by gel filtration on a Bio-Gel P-6 column. The chromatogram is shown in Fig. 5. Five fractions were isolated and analysed by ^{1}H NMR. Fraction E (20% w/w of the deacetylated A-8 alginate sample) was an unsaturated tri-mannuronate; Fraction D (5.5%) a mixture of pure oligomannuronates; and Fraction C (4.5%) contained both mannuronic and guluronic acid residues in the ratio M/G≅2. The ^{1}H NMR spectra (Fig. 6) of Fractions A (30%) and B (40%), agree well with an MG structure (M/G = 1). The average dp was estimated around 20 and 15, respectively, by integration of the characteristic ^{1}H signals: 5.08 ppm, H-1α of reducing-end; 4.95 and 4.60 ppm, H-1 and H-5 from guluronic units in MG sequences; and 4.56 ppm, H-1 from mannuronic units. From previous results on the pattern of substrate cleavage by mannuronate lyases, the unsaturated trimannuronate (ΔMM) is the final product and a catalytic site with 5 sub-sites has been proposed [26]. With regard to the present work, the

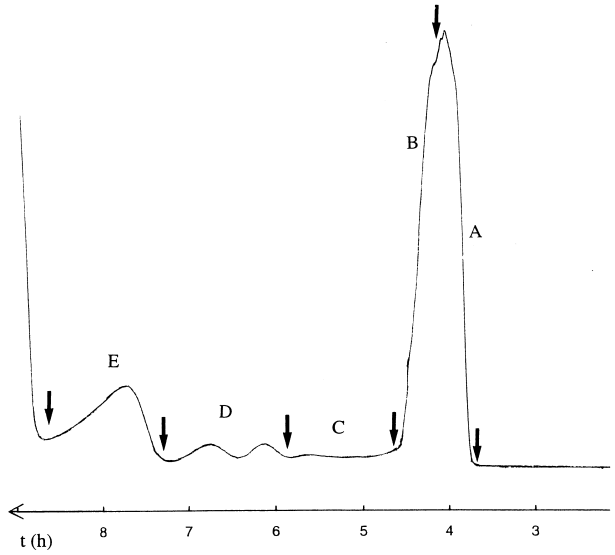

Fig. 5. Gel filtration on Bio-Gel P-6 of the AlxM_B-digest of deacetylated A-8 *Pseudomonas aeruginosa* alginate.

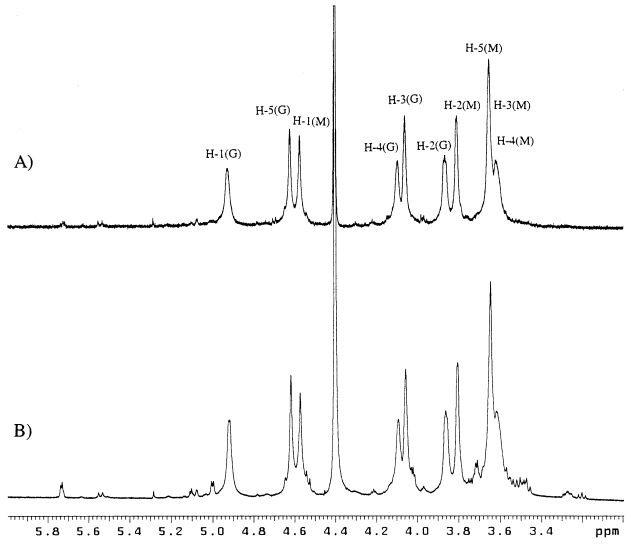

Fig. 6. ^{1}H NMR spectra at 50 °C of Fraction A (6-A) and B (6-B) from Fig. 5.

arrangement, $\Delta(MG)_n$ M, with $n \cong 10$, would be representative of the poly-MG blocks obtained in Fraction A (Fig. 6).

In conclusion, the judicious use of an enzyme, with a well known specificity on complex copolymers such as bacterial alginates, makes it possible to prepare substrates with a well defined structure. Such substrates could be used to determine the specificity of other alginate lyases. Conversely, the use of lyases with wellknown specificities on homopolymeric and alternating sequences of mannuronate and guluronate residues, should facilitate attempts to correlate the chemical compositions and monomer sequences of alginates with their gelling properties [30]. Furthermore, the controlled and specific degradation, by alginate lyases, of well-characterised alginates should permit the isolation of oligomeric substrates with predictable composition and degree of polymerisation for studies of their biological properties. It has been pointed out that the mannuronate lyases could be inhibited by degradation products such as heteropolymeric blocks [31]. By using the alternating-MG blocks presently available, the mechanism of inhibition by various alginate lyases should be re-investigated.

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