Biochemical analysis of the processive mechanism for epimerization of alginate by mannuronan C-5 epimerase AlgE4

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The enzymes mannuronan C-5 epimerases catalyse the in-chain epimerisation of β -D-mannuronic acid to α -L-guluronic acid in the last step of alginate biosynthesis. The recombinant C-5 epimerase AlgE4, encoded by the soil bacteria *Azotobacter vinelandii* and expressed in *Escherichia coli*, exhibits a non-random mode of action when acting on mannuronan and alginates of various monomeric compositions. The observed residue sequence has been suggested previously to be due to either a preferred attack or a processive mode of action. Based on methodologies involving specific degrading enzymes, NMR, electrospray ionisation mass spectrometry and capillary electrophoresis we show here that on

average 10 residues are epimerised for each enzyme–substrate encounter. A subsite model for the enzyme is analysed by the same methodology using native and ¹³C-labelled mannuronan oligomers as substrate for the AlgE4 epimerase. A hexameric oligomer is the minimum size to accommodate activity. For hexa-, hepta- and octameric substrates the third M residue from the non-reducing end is epimerised first.

Key words: alginate epimerization, electrospray ionization MS (ESI–MS), micellar electrokinetic capillary chromatography–UV (MEKC–UV), NMR, processive enzyme, subsite analysis.

INTRODUCTION

Alginate is a collective term for linear binary block copolymers of (1 → 4) α -L-guluronic acid (G residues) and (1 → 4) β -D-mannuronic acid residues (M residues) synthesised by brown algae and some bacteria. They are used extensively as gel-forming agents in food, pharmaceuticals and for cell immobilisation [1]. The composition and the sequential arrangements vary widely from homopolymeric mannuronan to polymers with > 70 % guluronic acid. Apart from mannuronan, most naturally occurring alginate does not exhibit a repeating unit and their sequence cannot be described by Bernoullian statistics. This non-random structure has been attributed to the post-polymerisation modification catalysed by mannuronan C-5 epimerases. These are enzymes that catalyse the conversion of mannuronic acid residues into guluronic residues in the polymer chain without breaking of the glycosidic bond, as shown in Figure 1. This reaction is analogous to the conversion of β -D-GlcA into α -L-IdoA in the biosynthesis of heparin, heparan sulphate and dermatan sulphate [2]. The alginate-producing bacterium *Azotobacter vinelandii* encodes seven secreted Ca²⁺-dependent epimerases, which have been sequenced, cloned and produced recombinantly in *Escherichia coli* [3,4]. Each of these recombinant enzymes generates specific non-random epimerisation patterns when acting upon mannuronan or alginate as substrate. The AlgE4 epimerase catalyses predominantly an alternating residue sequence, whereas the action of AlgE1, AlgE2 and AlgE6 generate long G blocks [4–9].

The following action patterns have traditionally been applied to describe enzymes that act on polymeric substrates: (1) the single-chain mechanism, in which the enzyme forms an enzyme–substrate (ES) complex, proceeds with performing its function

along the chain and does not dissociate from the polymer until the end of the chain or a non-convertible unit is reached, (2) the multi-chain mechanism, in which the enzyme reacts randomly with a polymer unit forming an ES complex, which dissociates after each reaction, and (3) the multiple-attack mechanism, which represents an intermediate of the two extremes by catalysing several reactions per effective encounter [10,11]. The three modes of action can be described more accurately in terms of enzyme processivity, which refers to the average number of times a reaction is repeated between association and dissociation of ES. Enzyme processivity influences whether single guluronate units are introduced at random, or introduced successively as blocks. The formation of long blocks (MG or GG) could thus be explained by a high degree of processivity.

High processivity is common for enzymes taking part in the replication and modification of RNA and DNA [12]; however, any enzyme that has more than one substrate-binding subsite, and performs multiple modifications on a substrate, may display processivity. Several examples of this type of action have been confirmed for polysaccharides, mainly for exolytic enzymes [11–15].

We have investigated previously whether the non-random pattern introduced by mannuronan C-5 epimerase is due to a processive mechanism, or rather is a consequence of a preferred reaction mechanism where the affinity for the substrates depends on pre-existing G residues. Kinetic experiments using time-resolved NMR spectroscopy and modelling based on Monte Carlo simulation suggest that AlgE4 is processive [6,7]. Recently we have reported on single molecular pair unbinding studies of mannuronan and AlgE4 and its modules using AFM (atomic force microscopy) [16]. The data are indicative of a processive mode of action from the ratio between the dissociation constant and

Abbreviations used: ABN, aminobenzonitrile; AFM, atomic force microscopy; CE, capillary electrophoresis; DP_n, degree of polymerisation; ES, enzyme–substrate; ESI–MS, electrospray ionisation mass spectrometry; F_G etc., molar fraction of guluronic acid etc.; G, α -L-guluronic acid; G_{red}, G-reducing end; M, β -D-mannuronic acid; M_{red}, M-reducing end; MEKC, micellar electrokinetic capillary chromatography; Δ , a 4-deoxy-L-erythro-hex-4-enopyranosyluronate residue at the non-reducing end (i.e. Δ G or Δ M).

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Figure 1 Epimerisation of a mannuronan segment with the mannuronan C-5 epimerase AlgE4

the catalytic constant for epimerising single hexose residues. However, detailed information about the 'extent' of processivity, the degree of polymerisation of the oligomer fully saturating the active cleft, and position of the converted residues in that sequence are still lacking. Here we use specific degrading enzymes and NMR spectroscopy, ESI-MS (electrospray ionisation mass spectrometry) and capillary electrophoresis (CE) to analyse the direction, the extent of processivity and the subsite specificity of AlgE4 when acting on a homopolymeric mannuronan.

MATERIALS AND METHODS

Alginates

High molecular mass mannuronan was isolated from the fermentation broth of an epimerase-negative strain [17] of *Pseudomonas fluorescens*. Purification and deacetylation were carried out as described previously [18]. No guluronate signals could be detected by ¹H-NMR (molar fraction of guluronic acid $F_G < 0.001$), indicating a homopolymeric mannuronan. An alginate sample rich in mannuronic acid ($F_G = 0.05$, and a molar fraction of the diad frequency $F_{GG} = 0.0$) was prepared from plate cultures of *Pseudomonas aeruginosa*, following the procedures for cultivation, deacetylation and purification reported previously [18]. Alginates with strictly alternating structures $F_G = 0.47$ and $F_{GG} = 0.0$, were prepared by incubating mannuronan with recombinant AlgE4 for 16 h [7]. Blocks with a high content of guluronic acid $F_G = 0.92$ and degree of polymerisation $DP_n > 20$ were prepared from *Laminaria hyperborea* stipes as described by Haug et al. [19]. Homopolymeric mannuronan samples of different number average DP_n were obtained by degrading the high-molecular mannuronan by partial acid hydrolysis at 95 °C (3.5 h at pH 5.6 and 6 h at pH 3.5). Following hydrolysis, the samples were neutralised (pH 7), and freeze-dried. Oligomers with DP_n from 2 to 12 were prepared by separating a hydrolysate by size exclusion chromatography on Superdex (Pharmacia, Uppsala, Sweden) column as described previously [20]. The same procedure was used to produce oligomers comprising only G or MG residues using polyG and polyMG respectively as starting material. In the latter case, since the GM linkage is more susceptible to acidic

hydrolysis than the MG linkage, the even numbered oligomers will predominantly have the same sequential structure with an M residue on the non-reducing and a G residue on the reducing end i.e. the hexamer MGMGMG.

A ¹³C-labelled mannuronan was produced by growing the mannuronan-producing *P. fluorescens* strain on agar plates with D-[1-¹³C]fructose (99%) as carbon source. The medium contained peptone (Oxoid L37) 20 g/l, MgCl₂ · 6H₂O 1.4 g/l, NaCl 5 g/l, D-[1-¹³C]fructose (Cambridge Isotope Laboratory, Massachusetts, U.S.A.), 3 g/l. The plates were incubated for 48 h at 20 °C and then for another 72 h at 6 °C. The polymer was harvested and purified and the oligomers prepared as described for the non-radio-labelled mannuronan above. The mannuronan product was selectively enriched to 59% ¹³C in the C-1 position [7].

Enzymes

The mannuronan C-5 epimerase AlgE4 with a molecular mass of 57.7 kDa was produced by fermentation of a recombinant *E. coli* strain JM 105 [5]. The enzyme was partly purified by ion-exchange chromatography on Q-Sepharose FF (Pharmacia) and by hydrophobic-interaction chromatography on phenyl Sepharose FF (Pharmacia). The activity of the enzyme was assayed by measuring the release of ³H to water, when ³H-5-labelled mannuronan was incubated with the enzyme [18]. A hybrid epimerase KA1, comprising a mixed A module from AlgE2 and AlgE4 was produced recombinantly in *E. coli* [21]. When acting upon mannuronan this enzyme introduces diad and triad frequencies, which fit Bernoullian statistics, and is therefore designated as random. Alginate α-L-guluronic acid endolyase (G-lyase) was isolated from *Klebsiella pneumoniae* as described in [22] and a specific β-D-mannuronic acid endolyase (M-lyase) from *A. vinelandii* [23] produced recombinantly in *E. coli* was kindly provided by Dr Helga Ertesvåg (Department of Biotechnology, NTNU, Trondheim, Norway).

Lyase degradation

Alginate (35 mg) was degraded by G- or M-lyase (5.56×10^{-2} units/mg alginate) in 50 mM Tris buffer, pH 7.5, with 0.5% NaCl for 1.5 h at 30 °C. The enzyme was then inactivated by heating the sample in a water bath at 95 °C for 5 min.

C-5 epimerisation of oligomers

Three solutions containing DP6 (3.4 mg/ml, 0.5 ml), DP7 and DP8 (4.0 mg/ml, 1 ml) dissolved in 50 mM ammonium acetate (pH 7.4), were mixed with 100 μl (DP6: 50 μl) of AlgE4 epimerase solution in the same buffer (2.5 mg/ml) and CaCl₂ (1 mM). The solutions were left at 37 °C for 12 h; 200 μl (DP6: 50 μl) were sampled for the CE analysis.

Degradation of epimerised oligomers with G-lyase

After reaction with epimerase, samples DP6 (0.45 ml), DP7 and DP8 (0.8 ml) were treated with G-lyase. The ionic strength was adjusted by adding NaCl to 0.5% and alginate G-lyase (100 μl; 0.3 unit/ml) was added. The solutions were incubated at 30 °C for 2 h. The enzyme was then inactivated at 90 °C for 5 min.

¹H-NMR spectroscopy

Spectra were recorded on a Bruker Avance DPX 300 or 400 spectrometer. To reduce the viscosity of the high molecular mass polymers for NMR analysis, the samples were degraded by mild acid hydrolysis [18] to a final DP_n of approx. 35. Aqueous solutions of 0.5 mg/ml alginate at pH 5.6 were kept for 60 min in a water bath at 95 °C, then the pH was adjusted to 3.8 and the solutions kept for 40 min at 95 °C.

Time-resolved ^1H -NMR

To monitor the progress of a single lyase experiment, series of several hundred successive ^1H -NMR spectra were recorded [7,8]. The procedure was automated by using the multi-zg command or the Bruker 'Icon NMR' automation software. Sample tubes with a diameter of 5 mm were used, and 3-(trimethylsilyl)-propionic-2,2,3,3- d_4 acid sodium salt (Aldrich, Milwaukee, WI, U.S.A.) was used as internal standard for the chemical shift. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was used as Ca^{2+} source for the enzyme. Spectra were obtained using a 30 degree pulse angle, a spectral width of 3612 Hz, and a 32 K data-block size; 64 scans were accumulated after eight dummy scans. The resulting time interval between two successive spectra was 8.5 min. The thermal stability of the lyase allowed us to work at 45–50 °C, and to avoid pre-saturation or other techniques to reduce the HDO (water/deuterium oxide) signal, in order to prevent distortion of neighbouring alginate signals.

ESI-MS

Mass spectra were recorded on an API-I PE SCIEX quadrupole mass spectrometer equipped with an articulated ion spray connected to a syringe pump for sample injection. The injection flow rate was equal to 0.1 ml/h; the electrospray voltage was –5 kV (negative mode) or 5.5 kV (positive mode). The analysed m/z range was 150–2400. The solvents were water or 50 % aqueous methanol containing formic acid (0.5 %) in positive mode and 50 % aqueous methanol containing ammonia (1 %) in negative mode.

CE

The system was an Applied Biosystems HPCE Model 270AHT with Turbochrom Navigator (4.0) software. The fused silica column [72 cm (50 cm to detector), 50 μm i.d. and 375 μm o.d.] was from Supelco (St. Louis, MO, U.S.A.). All runs were done at 27 °C. Samples were loaded under vacuum at a pressure of 16.9 kPa (1.5 s). Before sample injection, the capillary was washed for 4 min with buffer followed a 2 min washing with 0.1 M NaOH (vacuum pressure 67.6 kPa). Native unsaturated oligosaccharides were analysed with 50 mM tetraborate (pH 9) as the buffer (applied voltage 15 kV), with a detection wavelength equal to 232 nm. Derivatisation of the starting and epimerised oligomannuronate was accomplished by reaction with 4-aminobenzonitrile (4-ABN) (Aldrich, Milwaukee, WI) in the presence of NaCNBH_3 . Briefly, 50 μl of sample solution in water were added to 450 μl of a solution containing 4-ABN (0.5 M) and NaCNBH_3 (0.16 M) in methanol/acetic acid (19:1 v/v) [20]. The reaction was carried out for 15 min at 90 °C. Derivatisation mixtures were diluted five times with water and filtered through a 0.2 μm pore size membrane (Nylaflo, Aldrich, St. Louis, MO, U.S.A.) prior to injection into the CE system. The analysis was performed by micellar electrokinetic capillary chromatography (MEKC) [24]; the buffer was 660 mM boric acid, pH 8, containing 100 mM SDS. The applied voltage was 18 kV and the detection wavelength was 285 nm. The areas of the peaks divided by the migration time were used for quantitative calculations.

RESULTS AND DISCUSSION

Analysis of the distribution of epimerised sequences with specific alginate lyase

The epimerisation pattern of AlgE4 was studied by analysing the distribution of guluronate residues in the partly AlgE4-epimerised mannuronate product [5]. If AlgE4, an enzyme that introduces

Figure 2 Illustration of the degradation products produced upon partial degradation of different alginate sequences by a G-lyase

(A) G-lyase degradation of high M alginate with G residues in an alternating structure with M residues. (B) G-lyase degradation of alginate containing single G residues distributed randomly in the polymer.

predominantly single G residues (see Figure 1), works by a processive mode of action, the guluronate residues should be situated in strictly alternating MG sequences even for low degrees of epimerisation. The distribution of guluronate residues was investigated by degrading the epimerised alginates with alginate G-lyase. This lyase is specific for guluronic acid in GG and GM glycosidic linkages [25]. When the enzyme attacks a sequence of alternating M and G residues the following product would arise:

where G_{red} is a G-reducing end and Δ signifies a 4-deoxy-L-erythro-hex-4-enopyranosyluronate residue at the non-reducing end arising in this case from an M residue. As illustrated in Figure 2 the reaction product at the non-reducing end will thus differ depending on whether the G residues occur as single randomly distributed units or as part of a long strictly alternating MG block. In the former case ΔM would be dominant while in the latter case the non-reducing end would be ΔG . Since the epimerase only changes the identity of individual units in a pre-existing polymeric chain, the degree of processivity will have a pronounced effect on the distribution of the epimerised units. On a molecular level this will lead to compositional heterogeneity, at least for low to moderate degrees of conversion as in our study. When these molecules are digested with a G-lyase the molecular mass distribution of the product is expected to be bimodal.

NMR analysis of lyase-degraded alginates

To assign the NMR spectra of the lyase-degraded alginates, standard samples were prepared by degrading a homopolymeric mannuronan, a fully AlgE4-epimerised mannuronan ($F_{\text{G}} = 0.47$, $F_{\text{GG}} = 0.0$), and a G block ($F_{\text{G}} = 0.92$) with the G- and M-lyase. Characteristic signals observed in ^1H -NMR spectra from these alginates degraded by G- and M-lyase are shown in Figure 3. Peaks have been assigned by comparison of the spectra and according to reference [20,27–29]. The spectra of MG-alginate, degraded by G- and M-lyase, show that the polymer is degraded differently by the two enzymes. ΔG non-reducing ends and G-reducing ends (α and β anomers) appear in the sample degraded by G-lyase, while ΔM non-reducing ends and M-reducing ends (α and β anomers) appear in the spectra of MG-alginate degraded by M-lyase. Since proton 4 of the unsaturated ends shifts depending on the nearest neighbour, $\Delta_4 \text{G}$ and $\Delta_4 \text{M}$ can be quantified even for low degrees of epimerisation where the resonance signal from the anomer in an MGM sequence will be difficult to assess.

Figure 3 Comparison of ^1H -NMR (300 MHz) spectra of regular alginate structures degraded by recombinant M-lyase from *A. vinelandii* and by G-lyase from *K. pneumoniae*

Recorded at 90 °C, pD 6.8 in $^2\text{H}_2\text{O}$. (A) PolyG degraded by G-lyase, (B) PolyMG degraded by G-lyase (PolyG refers to alginate of $F_G = 0.92$, while PolyMG refers to alternating alginate of $F_G = 0.47$, $F_{GG} = 0.0$). (C) PolyMG degraded by M-lyase, (D) mannuronan ($F_M = 1.0$) degraded by M-lyase and (E) mannuronan degraded by G-lyase. $\Delta_4\text{M}$ and $\Delta_4\text{G}$ signals, produced upon lyase degradation, arise at 5.75–5.85 p.p.m. and 5.85–5.95 p.p.m., respectively.

The fraction of delta units was calculated from the intensities of $\Delta_4\text{G}$ and $\Delta_4\text{M}$ directly, due to overlap of $\Delta_1\text{G}$ and $\Delta_1\text{M}$ signals with reducing ends. Since the $\Delta_4\text{G}$ and $\Delta_4\text{M}$ protons have been reported to have much longer relaxation times than $\Delta_1\text{G}$ and $\Delta_1\text{M}$ protons [26] the calculations of $F_{\Delta G}$ and $F_{\Delta M}$ from integration of $\Delta_4\text{G}$ and $\Delta_4\text{M}$ should lead to underestimated values of $F_{\Delta G}$ and $F_{\Delta M}$. The $\Delta_4\text{G}$ and $\Delta_4\text{M}$ are therefore only used to calculate the relative ratio $\Delta\text{G}/\Delta\text{M}$. $F_{\Delta G}$ and $F_{\Delta M}$ denote the fraction of unsaturated uronate units at the non-reducing ends that are 1–4 linked to guluronate and mannuronate residues respectively.

To evaluate whether the G-lyase had any preference for the GM linkage in the MGMG sequence or the GM residues at the end of an alternating block (GMM), we monitored the degradation process by incubating the sample with lyase in the NMR tube in the spectrometer. The time-resolved spectra given in Figure 4 show that $\Delta_4\text{G}$ and $\Delta_4\text{M}$ resonance appear simultaneously, indicating that both linkages are degraded at the same rate.

Analysis of G-lyase digests of epimerised mannuronan

Mannuronan partly epimerised with Alge4 to yield polymers with a content of α -L-GulA in the range from 4 to 47% ($F_G = 0.04$ – 0.47), and native alginate from *P. aeruginosa* ($F_G = 0.05$) (four parallels) were degraded with G-lyase and analysed by ^1H -NMR spectroscopy. As a control for more randomly epimerised alginates, in addition to the native polymer from *P. aeruginosa*, we treated mannuronan with the hybrid epimerase KA1. This enzyme, which comprises a mixed A module from Alge2 and Alge4, has been shown previously to introduce diad sequences that fit a Bernoullian statistic [21].

Figure 4 Stacked plot of the ^1H -NMR spectra (400 MHz) of degradation of alginate from *P. aeruginosa* by G-lyase

P. aeruginosa wild-type ($F_G = 0.05$, $F_{GG} = 0.0$) was degraded by G-lyase (5.56×10^{-2} units/mg alginate) at 50 °C in $^2\text{H}_2\text{O}$, pD 7.5. (A) Selected spectra after 13 min to 10 h. (B) Close-up of the formation of $\Delta_4\text{G}$ and $\Delta_4\text{M}$ peaks in the range 5.76–6.04 p.p.m. Signals due to $\Delta_4\text{M}$ and $\Delta_4\text{G}$ produced upon lyase degradation arise at 5.76 p.p.m. and 5.86 p.p.m. respectively.

The fraction of internal guluronate residues (F_G), and the $\Delta_4\text{G}/\Delta_4\text{M}$ ratio of the samples after degradation were determined. Values are given in Table 1 and spectra are summarized in Figure 5 and compared with the ^1H -NMR spectrum of MG-alginate degraded by G-lyase under the same conditions. From the spectra in Figure 5 it is evident that all the samples have been degraded by the G-lyase. Only the fully epimerised sample ($F_G = 0.47$) displays a weak resonance for the G-1 in an MGM sequence at 5.1 p.p.m. In all the other samples the G residues are located exclusively on the reducing end and/or neighbouring an unsaturated residue (ΔG -) at the non-reducing end of the molecules.

To investigate the size distribution of the oligomers in the lysate, the degraded samples were fractionated by gel filtration on a Superdex column and analysed by NMR. Apart from the fully epimerised sample, which was degraded into only short oligomers with $\text{DP}_n \approx 3$, the partially epimerised mannuronans, as well as the *P. aeruginosa* alginate, gave a bimodal distribution with a major void peak comprising materials with a $\text{DP}_n > 25$ as well as a fraction of some low molecular mass material (results not shown). The NMR spectra of the two fractions (see Figure 6) showed that the high molecular mass fraction comprises long homopolymeric stretches of mannuronic acid with a G on the reducing end and ΔM on the non-reducing end. The low molecular mass fraction displayed end signals only from ΔG and G_{red} . ESI-MS analysis of the low molecular fraction identified dimers and tetramers as the main components, which was confirmed by NMR to be ΔG and ΔGMG .

As an example, in Figure 7 the mass spectrum of the lyase-degraded sample initially containing 47% α -L-guluronic acid is reported. The insert shows the MEKC-UV analysis of the same sample; the double bond detection wavelength (232 nm) and the migration times further confirmed the identity of the oligomers. The tetramer and dimer were isolated by gel filtration and then injected into the CE system for peak attribution. The sample with 10% G also comprises, in addition to dimers and tetramers,

Table 1 Molecular characteristics of epimerised alginates degraded by G-lyase

Δ_4G/Δ_4M and F_G of polyM samples of native and epimerised alginates degraded by G-lyase from *K. pneumoniae* (5.56×10^{-2} units/mg alginate). Samples were analysed by 1H -NMR (300 MHz) spectroscopy, in 2H_2O at 90 °C and pD 6.8. Samples ($F_G = 0.04$ – 0.47) were produced by epimerisation of mannuronan with AlgE4. The KA1 epimerised sample, refers to high M alginate with G residues distributed close to randomly throughout the polymer obtained by treating mannuronan with the hybrid epimerase KA1. PolyM from *P. aeruginosa* ($F_M = 0.95$) (four parallels) refers to native alginate. MM, molecular mass; epim., epimerised. n.d., not determined.

Sample	Initial F_G	Δ_4G/Δ_4M	$\Delta G/\Delta M$ Calculated For random Distribution*	Low MM Products	$\Delta G/\Delta GMG$	GM Length†
AlgE4 epim.	0.04	6.5	0.04	$\Delta G, \Delta GMG$	0.6	10
AlgE4 epim.	0.06	6.7	0.06	$\Delta G, \Delta GMG$	0.6	10
AlgE4 epim.	0.10	7.2	0.11	$\Delta G, \Delta GMG$	0.5	11
AlgE4 epim.	0.47	> 15	0.89	$\Delta G, \Delta GMG$	0.75	> 20
KA1 epim.	0.06	0.8	0.06	n.d.	n.d.	n.d.
<i>P. aeruginosa</i>	0.05	1.5 ± 0.2	0.05	n.d.	n.d.	n.d.

* See Appendix A.

† See Appendix B.

Figure 5 Comparison of 1H -NMR spectra of native and AlgE4 epimerised alginate samples degraded by G-lyase

Spectra of G-lyase degraded alginate from (A) *P. aeruginosa* wild-type $F_G = 0.05$ and mannuronan epimerised by AlgE4; (B) $F_G = 0.06$, (C) $F_G = 0.10$, (D) $F_G = 0.13$ are compared with the spectra of G-lyase degraded MG-alginate (E) $F_G = 0.47$ and (F) mannuronan epimerised with the hybrid enzyme KA1. A comparison of the section of the NMR spectra covering the Δ_4G and Δ_4M peaks at 5.74–5.98 p.p.m. for G-lyase treated polymers of (inset A) *P. aeruginosa* wild-type, (inset B) AlgE4 epimerised mannuronan $F_G = 0.06$, and (inset F) KA1 epimerised mannuronan $F_G = 0.06$ is shown.

traces of pentamers and probably also some oligomers with higher molecular mass. Taken together, the bimodal distribution and the identity of reducing and non-reducing end groups are consistent with the model suggested in Figure 2(A), and thus are indicative of a processive action of AlgE4.

Degree of processivity reflected by the Δ_4G/Δ_4M ratio

The most conspicuous difference in the G-lyase degradation pattern between the native alginates and the epimerised mannuronans is visualized by the Δ_4G/Δ_4M ratio (Figure 5 and Table 1).

Figure 6 1H -NMR spectra (300 MHz) of a partly AlgE4 epimerised mannuronan ($F_G = 0.10$) degraded with G-lyase and fractionated by gel filtration on Superdex 30

(A) The void fraction and (B) the low molecular mass fraction.

The Δ_4G/Δ_4M ratios range from 6–7 for the partly epimerised mannuronan, to 1.5 for *P. aeruginosa* polymers and 0.8 for the KA1 epimerised mannuronan. This variation reflects the different lengths of the alternating sequences in the three types of polymers. The ratios are much higher than would be expected if the G units were distributed randomly, and indicate long sequences of repeated MG. The theoretical ratio $\Delta G/\Delta M$ for a complete degradation of a polymer with a Bernoullian distribution of G residues is given simply by F_G/F_M (for the derivation of the equation see Appendix A). This implies that for a random polymer with 10% G the theoretical ratio $\Delta G/\Delta M \approx 0.1$. This observed discrepancy suggests that not only the AlgE4 epimerised samples, but also the wild-type alginates and KA1 epimerised mannuronan have a non-random distribution of G residues. As positive control we used a fully epimerised sample $F_G = 0.47$ where the Δ_4G/Δ_4M is larger than 15.

Calculation of the average length of the GMG blocks

To calculate the length of the repeated GM sequence we cannot use the $\Delta G/\Delta M$ ratio alone, but must also account for the incomplete depolymerisation by G-lyase using both the experimentally determined ratios of $[\Delta G]$ to $[\Delta M]$ by NMR and the molar ratio of dimers and tetramers in the final products.

Figure 7 Electrospray mass spectrum of 47% guluronic acid sample after G-lyase digestion

ionspray voltage: +5600 V; solvent: aqueous 50% CH₃OH, containing 0.5% HCOOH; peak attributions: unsaturated dimers, 375.2 [M + Na]⁺ and 391.0 [M + K]⁺; unsaturated tetramer, 727.1 [M + Na]⁺ and 743.2 [M + K]⁺. In the inset: CE analysis of the same mixture (underivatised); buffer: 50 mM sodium tetraborate; 15 kV; fused silica capillary.

The average length of the alternating sequences was calculated from the ratio $\Delta G/\Delta M$ and the molar ratio of the oligomers $[\Delta G]/[\Delta GMG]$ for the G-lyase-digested samples (Appendix B):

$$\langle GM \rangle_{\text{Length}} = \frac{[\Delta G^-]_{\text{NMR}}}{[\Delta M^-]_{\text{NMR}}} \left(1 + \frac{1}{[di]/[tetra] + 1} \right) + 1$$

Where $\langle GM \rangle_{\text{Length}}$ signifies the average number of repeating units in the MG blocks and $[\Delta G]/[\Delta GMG]$ is the molar ratio determined from CE analysis. The data given in Table 1 show that even at the lowest degree of epimerisation (4%) long MG sequences are introduced, suggesting that the AlgE4 enzyme slides along the mannuronan chain until an average of 10 residues are epimerised. The β -1,4 linkage in mannuronan implies that each residue is rotated nearly 180° with respect to its neighbours. Thus, by epimerising every second residue, the enzyme can slide along the substrate chain in a processive manner without rotation.

Subsite specificity

A polymer-modifying enzyme that acts in a processive mode would have to interact with more than one residue in the polymer substrate. By using [5-³H]-labelled alginate oligomers we have demonstrated previously that the octamer was the minimum size necessary to support enzyme activity of AlgE4 [6]. In these experiments we did not have access to homogeneous mannuronate oligomers. Based on these studies we suggested a subsite model for AlgE4, which needed at least four sub-sites to account for the processive mode of action, or preferred attack yielding the epimer-

Figure 8 MEKC-UV analysis of ABN-derivatised hexamer (0.1 mg/ml) (A), heptamer (0.3 mg/ml) (B) and octamer (0.25 mg/ml) (C) before enzymic treatment

*From ABN; other experimental conditions are reported in the text.

isation pattern found by NMR [5]. An increase in the number of subsites was not included in the mathematical modelling because of the increase in the number of parameter values needed to be determined [5]. To test such a subsite model in more detail we have epimerised pure mannuronate oligomers with AlgE4 and investigated the presence and position of any epimerised residue by degrading the oligomers with the specific G-lyase. By analysing the distribution of the oligomers by ESI-MS and CE, it was possible to identify the position of the epimerised units.

C-5 epimerisation of oligomers

Hexamannuronic acid (DP6), heptamannuronic acid (DP7) and octamannuronic acid (DP8) isolated by size-exclusion chromatography from a mannuronan hydrolysis mixture, were used as well-defined substrates for biochemical subsite studies of AlgE4. In Figure 8 (panels A, B, and C respectively) the MEKC-UV analysis of the three substrates (derivatised with ABN) before enzymic treatment is reported. It can be clearly seen that all these fractionated samples also contain the $(n+1)$ oligomer, as confirmed also by ESI-MS (results not shown).

The electropherograms of the epimerised samples (derivatised with ABN) are reported in Figure 9. The MEKC-UV profile of hexamer (Figure 9A) has a very similar appearance relative to the unreacted sample, also in terms of ratio between the molar concentrations of the components. No significant new compounds could be detected by MEKC-UV. This means that the AlgE4 acts poorly on hexa- (and hepta-) mannuronic acid. The ESI-MS analysis of the sample subsequently treated with G-lyase (see below), however, provided evidence that some epimerisation occurred also on the hexamer.

In the case of DP7 (Figure 9B), treatment with epimerase induces the presence of some new compounds and a relatively high decrease of the amount of octamer with respect to heptamer.

Figure 9 MEKC-UV analysis of ABN-derivatised hexamer (A), heptamer (B) and octamer (C) after treatment with epimerase

Experimental conditions are reported in the text.

These results indicate that the efficiency of the epimerase action is higher for the octamer than for the heptamer. The new peaks are attributed to the hepta- and octa-uronic acids containing guluronic acid units. The increase of AlgE4 efficiency with the number of monomeric units is confirmed by the CE analysis of the epimerised octamer shown in Figure 9(C), where three new peaks were clearly detected. The intensity of the octa- and nonamer peaks decreased in a similar way due to epimerisation.

Degradation of epimerised oligomers with G-lyase

Some decisive information concerning the AlgE4 mode of action was obtained upon treatment of the samples with G-lyase and subsequent analysis by ESI-MS. In Figure 10 the mass spectra (negative mode) of three samples are reported. In all cases, a signal attributable to a trimer without unsaturations ($m/z = 545$) could be clearly seen. In the case of *DP6* (Figure 10A), there is still a detectable amount of the unreacted hexamer, as found by CE; the presence of the peak at $m/z = 527.0$, attributable to a *DP3* with one unsaturation, however, demonstrates that the epimerase can also act weakly on a hexamer. The peak at 351.2, relative to *DP4* with an unsaturation, could originate from the degradation of the heptamer contained in the *DP6* sample. Epimerisation of a hexamer was also verified by purifying the hexamer to homogeneity (98 %) by repeating size exclusion chromatography on Superdex and recording the NMR spectra and the G-lyase degradation pattern. Non-epimerised M-oligomers used as negative control, were not degraded by the G-lyase.

In the case of *DP7* and *DP8* (Figures 10B and 10C, respectively), in addition to the trimer (without unsaturations), other peaks attributable to the oligouronates containing double bonds could be observed, which undergo multiple-charging phenomena during the ionisation process. Peak assignments are reported in the Figure. The unreacted heptamer, octamer (present in *DP7* and *DP8* samples) and nonamer (in *DP8* sample) were not detected.

Figure 10 Electrospray mass spectra of hexamer (A), heptamer (B), and octamer (C) after treatment with epimerase and G-lyase

Peak attributions: (A) unsaturated tetramer, 351.2 $[(M-2H^+)/2]^{2-}$, unsaturated trimer, 527.3 $[M-H]^-$, saturated trimer, 545.0 $[M-H]^-$, saturated (unreacted) hexamer, 1073.3 $[M-H]^-$; (B) unsaturated pentamer, 461.2 $[(M+2Na^+-4H^+)/2]^{2-}$, unsaturated tetramer, 373.5 $[(M+2Na^+-4H^+)/2]^{2-}$, saturated trimer, 545.4 $[M-H]^-$; (C) unsaturated pentamer, 460.8 $[(M+2Na^+-4H^+)/2]^{2-}$, saturated trimer, 544.8 $[M-H]^-$; ionspray voltage: -5 kV; solvent: aqueous 50 % CH_3OH , containing 1 % NH_3 .

All the ESI-MS data in Figure 10 indicate that AlgE4 acts on the third sugar in an oligomeric chain with *DP* equal or higher than 6, regardless of the length (6, 7, 8 or 9 monomeric units) although AlgE4 activity increases with the chain length. As a control we treated the two hexamers GGGGGG and MGMGMG with G-lyase and analysed the fragments by MS. In the former case GG and Δ GGG were the dominating products, while the latter produced mainly the saturated dimer MG and the unsaturated tetramer Δ GGMG, but the tetramer MGMG and the dimer Δ G were also detected (Table 2). This shows that the G-lyase activity is not limited to a G residue in position 3, but actually can split the glycosidic linkage after a G residue in both positions 2 and 4.

^{13}C -NMR analysis of epimerised oligomers

To establish if more than one residue could be epimerised in each oligomer we searched for the GMG triad by using ^{13}C -NMR.

Table 2 The composition and G-lyase degradation pattern of AlgE4 epimerised ¹³C-labelled mannuronate oligomers

F_G and F_{GMG} were calculated by integration of ¹³C-NMR spectra (100 MHz) of the AlgE4 epimerised C-1 ¹³C-labelled mannuronate oligomers. The size and composition of saturated and unsaturated oligomers in the G-lyase digests of the samples were obtained by ESI-MS; ionspray voltage: +5600 V. Oligomers of regular alginate structures (pure M, G and MG oligomers) were used as a control of the specificity of the G-lyase for G residues in an alginate chain (GG or GM glycosidic linkages), regardless of the position of the G residues within the chain. Minor peaks are given in parentheses.

Mannuronate oligomer	F_G	F_{GMG}	Number of residues in saturated oligomers after G-lyase digestion (MS)	Number of residues in unsaturated oligomers after G-lyase digestion (MS)
Hexamer	0.04	n.d.	3 and 6	Δ3 (Δ2 and Δ4)
Heptamer	0.12	0.04	3 (7)	Δ4 (Δ2 and Δ5)
Octamer	0.22	0.12	3	Δ5 (Δ2 and Δ3)
Nonamer	0.02	n.d.	3 and 9	Δ6 (Δ4)
Nonamer	0.25	0.14	3 (5)	Δ2, Δ4 and Δ6 (Δ3)
Controls:				
MMMMMMM	0	0	7	0
GGGGG	1	0	2 (3 and 4)	Δ4 (Δ2 and Δ3)
MGMGMG	0.5	0.5	2 (4)	Δ4 (Δ2)

Figure 11 ¹³C-NMR spectra of C-1 ¹³C-labelled mannuronan octamer epimerised with AlgE4 for 24 h

The lower spectrum was obtained on the mannuronan oligomer before epimerisation, the upper after epimerisation. The spectra of 3.5 mg/ml were recorded on a Bruker DPX 400 spectrometer.

Oligomers of DP6, DP7, DP8 and DP9 (4 mg), prepared from a C-1 ¹³C-enriched mannuronan, were epimerised for 24 h. The spectra of the epimerised octamer ($F_G = 0.22$) (Figure 11) clearly displayed resonance peaks for the three M-centred triads MMG, GMM and most interestingly GMG. The intensity of the resonance from GMG was similar to the intensity of the resonance from MMG, and together they summed up to the intensity of the G centred triad MGM. This suggests an octamer with the sequence MMGMGMMM as a dominating structure, although the slightly elevated content of MMM could be explained by unreacted oligomers and by contamination with the nonamer. This sequential structure was also supported by ESI-MS. When the sample was degraded with G-lyase, the dominating fragments comprise a saturated trimer, an unsaturated dimer (ΔG) and an unsaturated trimer (ΔMM) as suggested in Figure 12. The composition of the epimerised oligomers and the G-lyase degradation pattern are given in Table 2. For the hexamer (4% G residues) only MMG was detected while in the heptamer ($F_G = 0.11$) the GMG resonance was very weak compared to MMG. The GMG triads found in the heptamer probably originate from the contaminating octamer (20%). This suggests that the epimerase is inefficient in introducing a second G residue into oligomers smaller than

Figure 12 Tentative degradation pattern for G-lyase acting upon an epimerised octamer

(A) One residue epimerised and (B) two residues epimerised.

Figure 13 Tentative subsite model for AlgE4 for two consecutive epimerisations on an octamer

The arrow indicates the direction of the processive action. The consecutive subsites are depicted with increasing numbers, and the residue that is converted is bound to subsite +1.

8 units. This fits with a subsite model with six binding sites if we assume that the epimerisation will occur only on substrates that saturate all the subsites as illustrated in Figure 13.

To study the direction of processive movement of the enzyme a ¹³C-labelled nonamer was epimerised for 10 min and 24 h, giving 2% and 24% G respectively. The subsequent degradation of the two samples with G-lyase produced only saturated trimers and the corresponding hexamer from the former 10 min sample, while the 24 h sample showed a more complex mixture comprising both saturated trimers and pentamers. This suggests, at least on oligomeric substrates, that the enzyme acts from the non-reducing end towards the reducing end.

Concluding remarks

Although the three-dimensional structure of the epimerase is currently lacking, the present results suggest an active cleft of the epimerase which has several subsites. An extended active cleft, which binds to a number of consecutive residues, is a common feature for processive enzymes [14]. This is also supported by the reported crystal structure of an alginate lyase where the active cleft accommodates at least six residues [30]. The specificity of each subsite in the epimerase AlgE4 has not been fully mapped, but some information has emerged from this and previous studies. The catalytic site (+1) as well as the two flanking sites (−1) and (+2) bind β-D-mannuronate residues. In a recent study on the action of the epimerase on C-6 oxidized glucomannans the identification of a GlucA-α-L-(1 → 4)GulA β-D-(1 → 4) ManA sequence indicates that subsite (−1) can also bind to β-D-glucuronic acid [31]. Site (−2) may accommodate ManA as well as GulA residues while the specificity of site (+3) and (+4) has not been investigated. The subsite specificity of the other epimerases in the AlgE family and the crystal structure of AlgE4 are currently being investigated in our laboratory.

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APPENDIX A

The theoretical ratio $\Delta G/\Delta M$ for any given alginate with a random distribution of G residues degraded by a G-lyase can be calculated provided that every GG and GM linkage is broken by the lyase. Generally only the two triad sequences GMG and GGG can give rise to a ΔG , while GMM and GGM give ΔM . $\Delta G/\Delta M$ is thus given by the molar ratio:

$$\Delta G/\Delta M = (F_{GMG} + F_{GGG})/(F_{GMM} + F_{GGM}) \quad (A1)$$

For any given random composition the $\Delta G/\Delta M$ is given by:

$$\Delta G/\Delta M = F_G^2 F_M + F_G^3 / F_G F_M^2 + F_G^2 F_M \quad (A2)$$

Recasting this to:

$$\Delta G/\Delta M = F_G(F_M + F_G)/F_M(F_M + F_G) \quad (A3)$$

Since $(F_M + F_G) = 1$

$$\Delta G/\Delta M = F_G/F_M \quad (A4)$$

APPENDIX B

The observed NMR signals of the ΔG and ΔM of the G-lyase depolymerised alginates were used as a basis for calculation of the number average length of the GM-type sequences as follows. The fact that G-lyase cannot depolymerise tetramers and shorter oligomers needs to be taken into account. The present analysis applies only to alternating sequences, i.e. those being introduced by epimerase AlgE4, where the assumption that all tetramers are of the type ΔGMG holds at low degree of conversion.

The molar concentrations of ΔG and ΔM determined by NMR are given by the molar concentration of dimers, tetramers and other sequences:

$$[\Delta G]_{\text{NMR}} = [\Delta G] + [\Delta GMG] \quad (B1)$$

$$[\Delta M]_{\text{NMR}} = \sum_i [\Delta M_i G] + \sum_j [\Delta M_j] \quad (B2)$$

The terms in eqn (B1) arise from G-lyase depolymerisation of the alternating sequences. The first term in eqn (B2) reflects mannuronan sequences between alternating sequences and the last term is the terminal part. Note that the signal can be viewed as the termination signal of the strictly alternating residue sequence independent of the length of the mannuronan sequences and distribution between the two parts. The number average length of the strictly alternating sequence is given by:

$$\langle GM \rangle_{\text{Length}} = \frac{[\Delta G]_{\text{Tot}}}{[\Delta M]_{\text{Tot}}} + 1 \quad (B3)$$

Since tetramers are not depolymerised by G-lyase, the following relation holds:

$$[\Delta G]_{\text{Tot}} = [\Delta G] + 2[\Delta GMG] \quad (B4)$$

Insertion of eqn (B2) in (B4) yields:

$$[\Delta G]_{\text{Tot}} = [\Delta G]_{\text{NMR}} + [\Delta GMG] \quad (B5)$$

Realising that $[\Delta M]_{\text{Tot}} = [\Delta M]_{\text{NMR}}$, yields the following expression for the length of the alternating sequence:

$$\begin{aligned} \langle GM \rangle_{\text{Length}} &= \frac{[\Delta G]_{\text{Tot}}}{[\Delta M]_{\text{Tot}}} + 1 \\ &= \frac{[\Delta G]_{\text{NMR}}}{[\Delta M]_{\text{NMR}}} \left(1 + \frac{[\Delta GMG]}{[\Delta G] + [\Delta GMG]} \right) + 1 \quad (B6) \end{aligned}$$

Recasting this to:

$$\langle GM \rangle_{\text{Length}} = \frac{[\Delta G]_{\text{NMR}}}{[\Delta M]_{\text{NMR}}} \left(1 + \frac{1}{[\Delta G]/[\Delta GMG] + 1} \right) + 1 \quad (B7)$$

allows correction for incomplete depolymerisation by G-lyase of alternating sequences using both the experimentally determined ratios of $[\Delta G]_{\text{NMR}}/[\Delta M]_{\text{NMR}}$ and the ratio of dimers and tetramers in the final products.

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