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HPLC analysis of saturated or unsaturated oligoguluronates and oligomannuronates. Application to the determination of the action pattern of *Haliotis tuberculata* alginate lyase

Alain Heyraud [a,*], Philippe Colin-Morel [a], Sylvie Girond [b], Christophe Richard [b], Bernard Kloareg [b]

[a] *Centre de Recherches sur les Macromolécules Végétales, C.N.R.S. and University Joseph Fourier, B.P. 53, F-38041 Grenoble, France*

[b] *Centre d'Etudes d'Océanologie et de Biologie marine, C.N.R.S., B.P. 74, F-29682 Roscoff, France*

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Abstract

The chromatographic behaviour of various saturated and unsaturated oligouronates obtained by acid or enzymatic degradation of homopolymeric blocks of alginates was investigated by isocratic anion exchange liquid chromatography. This approach was then applied to the determination of the catalytic properties of *Haliotis tuberculata* alginate lyase. This enzyme presents a high affinity for poly-β-D-mannuronate blocks, leading to the release of *O*-(4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronic acid)-(1 → 4)-*O*-(β-D-mannopyranosyluronic acid)-(1 → 4)-*O*-β-D-mannopyranuronic acid as the main end reaction product. Kinetic analysis with oligomannuronates of various sizes indicate that the catalytic site of *Haliotis tuberculata* lyase (abalone) best accommodates an oligomannuronate pentamer. The abalone lyase, however, is also capable of cleaving the G–M linkages of alginate heteropolymeric sequences. In contrast, it does not degrade the G–G nor the M–G diads. This lyase should therefore be referred to as a mannuronate β-eliminase, indicating that the enzyme performs β-elimination on mannuronate residues only, from both the M–M and G–M diads of alginates. © 1996 Elsevier Science Ltd.

Keywords: *Haliotis tuberculata*; Alginate lyase; Oligo-alginates; HPLC

* Corresponding author.

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1. Introduction

Alginates are linear anionic copolymers of β-(1 → 4)-D-mannuronic acid and of its C-5 epimer, α-(1 → 4)-L-guluronic acid, produced by marine brown algae and by a limited range of Gram negative bacteria. They consist of alternate homopolymeric blocks of poly-β-(1 → 4)-D-mannuronic acid (M), and poly-α-(1 → 4)-L-guluronic acid (G), and of heteropolymeric blocks with random arrangements of both monomers [1,2]. Because of their texturing properties in aqueous solution, alginates are widely used as gel-forming or viscosifying agents in various industrial applications. Depolymerized alginates are also considered for new applications based on their biological activity [3–9].

A number of alginate-depolymerizing enzymes have been described from marine algae, molluscs and bacterial sources [10–19]. They are lyases, which depolymerize alginate by a three-stage β-elimination reaction, similar in mechanism to the alkaline degradation of glycuronans [20,21], i.e. (i) the carboxyl group of the substrate is neutralized, possibly by formation of a salt bridge with a positively charged amino acyl side chain in the active site of the enzyme; (ii) a general base-catalyzed abstraction of the proton at C-5 of the uronic acid occurs, with formation of a resonance-stabilized enolate intermediate; (iii) a transfer of electrons from the carboxyl group to form a double bond between C-4 and C-5 results in the elimination of the 4-*O*-glycosidic bond and in the formation of 4-deoxy-L-*erythro*-hex-4-enopyranosyluronate. A consequence of this reaction mechanism is that an unsaturated uronate is produced at the newly generated, non-reducing terminal groups. Therefore, it is not possible to identify the uronic acid which was originally attached to O-4 of the cleaved glycosidic bond.

As alginates can be described by a combination of four different diads, M–M, M–G, G–M, and G–G, alginate lyase specificity has to be considered in terms of the relative rates of cleavage of each diad. In this respect, little is known about the exact specificity of alginate lyases. They are referred to as 'mannuronate- or guluronate-specific lyases' as well as 'polymannuronate or polyguluronate lyases', reflecting differences in their interactions with alginates with various mannuronate to guluronate ratios or with purified homopolymeric alginate blocks. This nomenclature, however, does not precisely indicate which uronic residue is primarily involved in the β-elimination process, nor specifies the influence of its neighbours on the elimination cleavage of alginates.

Information about substrate specificities, and the mode of substrate cleavage by alginate lyases, requires both the availability of oligo-alginates of known sequences as well as a non-ambiguous method for the analysis of lyase products. Ion-exchange and ion-pair high performance liquid chromatography have been developed for the analytical separation of oligo-alginates [22–25] and this approach was applied to the kinetic analysis of the degradation of M-blocks by a bacterial alginate lyase [22]. In spite of the improvement of chromatographic phases, however, no comprehensive data are available so far for the HPLC determination of more complex oligo-alginate sequences. Recently, we reported on the preparation of homologous series of saturated and unsaturated oligo-mannuronates and -guluronates and on their characterization by ^{1}H and ^{13}C NMR spectroscopy [26]. We have taken advantage of the availability of these oligo-alginates with a well-defined structure to reinvestigate the chromatographic behaviour of oligo-al-

ginate sequences. In association with liquid chromatography and ^{1}H NMR spectroscopy, this approach was then applied to the analysis of the action pattern of *Haliotis tuberculata* alginate lyase. This enzyme, initially described as a mannuronate lyase [27], was shown to act on mannuronic acid residues independently of their immediately surrounding neighbours [26,28–30]. In this work, we confirm that *Haliotis tuberculata* alginate lyase is a mannuronate β-eliminase, i.e., it is capable of performing β-elimination on the mannuronate residues from both the M–M and G–M diads of alginates.

2. Materials and methods

Preparation of alginate lyases.—*Haliotis tuberculata* alginate lyase was isolated from abalones collected at Roscoff, Brittany, France, and was purified by CMC-exchange chromatography according to Boyen et al. [27]. Guluronate lyase was isolated according to Lange et al. [10] from the culture broth of *Klebsiella pneumoniae*, kindly provided by K. Østgaard et al. [18]. Alginate lyase activity was measured by monitoring the increase in absorbance at 235 nm and 20 °C of a 2 mL reaction mixture containing 0.25% (w/v) sodium alginate from *M. pyrifera*, 10 mM Tris–MES buffer (pH 7.5), 100 mM NaCl, 20 mM $MgCl_2$, and 1–50 μL enzyme. One unit (U) of activity is defined as an increase of 1 unit per min in the absorbance at 235 nm.

Substrates.—Sodium alginates from *Laminaria digitata* fronds and from *L. hyperborea* stipes were kindly provided by Sanofi BioIndustrie (S.B.I., Carentan, France) and Sobalg (Landerneau, France). Sodium alginates from *Macrocystis pyrifera* were purchased from Sigma. Polyguluronate (G-blocks) and polymannuronate (M-blocks) homopolymeric blocks were prepared by two cycles of HCl-hydrolysis of *L. digitata* and *L. hyperborea* alginates, respectively, according to the procedure of Haug et al. [31]. The purity of homopolymeric block was assessed by ^{1}H NMR analysis [26]. M-Blocks contained 5–10% guluronate and G-blocks contained about 5% mannuronate. Saturated oligo-mannuronates and -guluronates were obtained by acid hydrolysis at 100 °C, for 5 h and 7 h, respectively, of a 0.1% (w/v) solution of the appropriate homopolymeric blocks in 10^{-3} M HCl. Unsaturated oligomannuronates were obtained by action of *Haliotis tuberculata* alginate lyase [27] on M-blocks (12 U/100 mg; 0.1% solution; 5 h at 25 °C). Unsaturated oligoguluronates were prepared by hydrolysis of G-blocks by *Klebsiella pneumoniae* alginate lyase (15 U/100 mg; 0.1% in 50 mM Tris–HCl, pH 7.5 solution; 5 h at 20 °C). The four oligo-alginate homologous series were then size-fractionated by gel filtration chromatography, on a 100 × 4.5 cm Bio-Gel P-6 column [23] eluted with $5 \cdot 10^{-2}$ M $NaNO_3$ solution at a rate of 90 mL h^{-1}, desalted and characterized by ^{1}H and ^{13}C NMR spectroscopy as reported previously [26].

High performance liquid chromatography of oligo-alginates.—Analytical chromatographic separation of the oligo-uronides obtained by acid and enzymic hydrolysis of alginate homopolymeric blocks was performed by anion exchange liquid chromatography, using a SB Nucleosil 5 μm prepacked column (250 × 4.6 mm) from Interchim (France). Chromatography was run isocratically at room temperature, with $NaNO_3$ solutions at a flow rate of 0.6 mL min^{-1}. Depending on the oligomer series, and in order to achieve optimal resolution, the eluent ionic strength was varied from 160–240 mM. Detection was performed on line with a R-410 Waters refractometric detector and a

Model 481 UV LC Spectrophotometer operating at 254 nm. Chromatograms were analyzed and recorded with a C-R4A chromatopac integrator (Shimadzu). The chromatographic behaviour of oligo-alginates was characterized by the capacity factor K', defined as the ratio $(t_e - t_o)/t_o$, where t_e refers to the elution time of oligo-uronates and t_o to the column void volume.

Analysis of alginate lyase products.—For the determination of the cleavage site of alginate by *Haliotis tuberculata* alginate lyase, various purified oligomannuronates (10 mM) were incubated at 20 °C in the presence of the lyase (0.2 U/mL). Aliquots (25 μL) were withdrawn at intervals and hydrolysis products were analyzed by HPLC. Preparative size-fractionation of the reaction products of *Haliotis tuberculata* lyase on *Macrocystis pyrifera* alginate was performed by gel filtration with a Bio-Gel P-6 column. Each fraction corresponding to a given dp was desalted on a 210 × 2.5 cm Bio-Gel P-2 column and analyzed by HPLC, showing evidence of the presence within each dp class of several, structurally-different oligoalginates. The various oligo-alginates identified by HPLC were then purified on a preparative Nucleosil 5 μm SB column (250 × 22 mm) and characterized by ^{1}H NMR, as described previously [26].

3. Results and discussion

High performance liquid chromatography of oligo-alginates.—HPLC separations with sodium nitrate on a Nucleosil 5 μm SB column of saturated or unsaturated

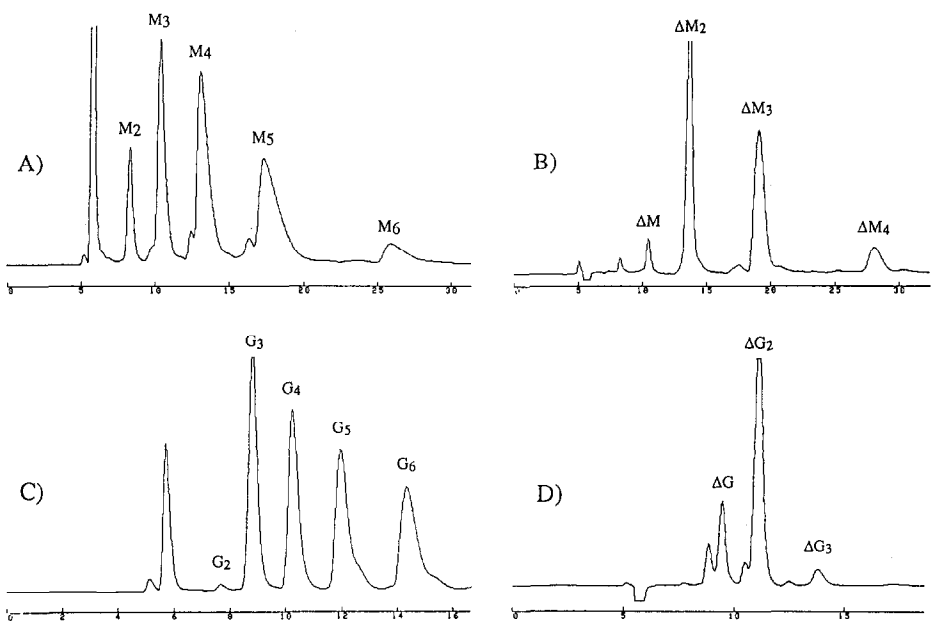

Fig. 1. High performance liquid chromatography separation of saturated and unsaturated oligo-alginates with 200 mM sodium nitrate on a Nucleosil 5 μm SB column. Flow rate was 0.6 mL min^{-1}; refractive index (RI) detection. (A) Oligomannuronates; (B) unsaturated oligomannuronates; (C) oligoguluronates; (D) unsaturated oligoguluronates.

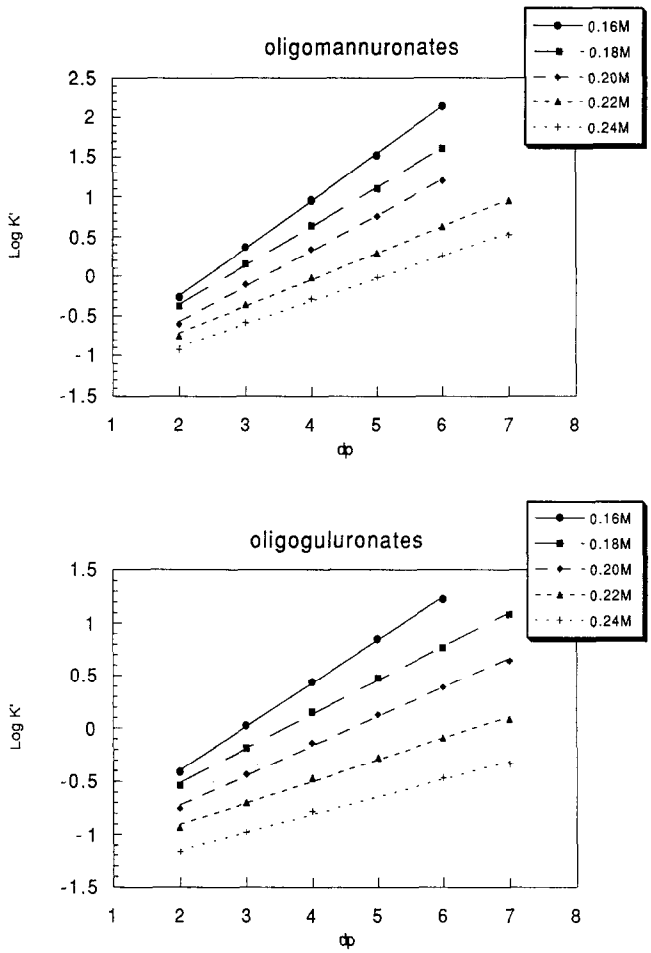

Fig. 2. Influence of the dp and of the eluent ionic strength on the capacity factor of saturated oligo-mannuronates and -guluronates. Nucleosil 5 μm SB column; the eluent was $NaNO_3$. The capacity factor, K', is defined as the ratio $(t_e - t_o)/t_o$, where t_e refers to the elution time of oligo-uronates and t_o to the column void volume.

oligomannuronates and oligoguluronates are shown in Fig. 1. As expected, in ion-exchange chromatography of oligo-uronides [24], the capacity factor (K') of oligo-alginates decreased with the eluent ionic strength (Fig. 2). A similar result was observed with the unsaturated oligomannuronates and oligoguluronates (not shown). Within each series, oligo-uronates were well resolved according to their dp. However, the chromatographic behaviour of oligo-uronates was strongly dependent on their fine structure (Fig. 3). For a given dp, oligoguluronates were less retained than oligomannuronates, a result which is likely to be related to the differences in their linkage configuration. It is also worth noting that, relative to the saturated series, oligo-uronates with an unsaturated ring were markedly delayed, to the point that the retention of an unsaturated oligomer was

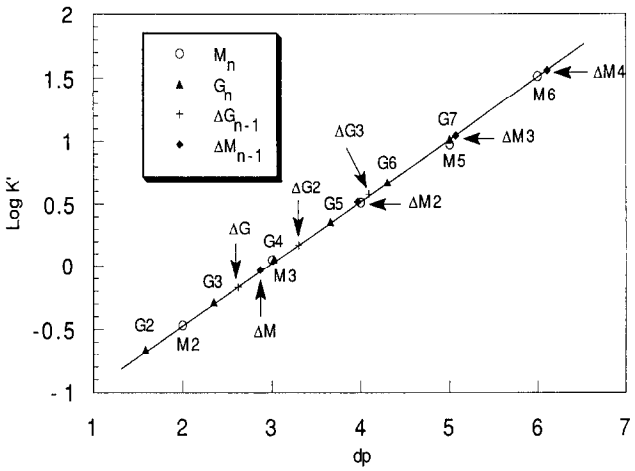

Fig. 3. Size and structure dependence of the capacity factor of saturated and unsaturated oligo-alginates. Nucleosil 5 μm SB column; flow rate, 0.6 mL min^{-1}; the eluent was 200 mM $NaNO_3$. Δ Refers to 4-deoxy-L-*erythro*-hex-4-enopyranosyluronate, *n* to the oligo-uronate dp and indices indicate the number of saturated uronic residues.

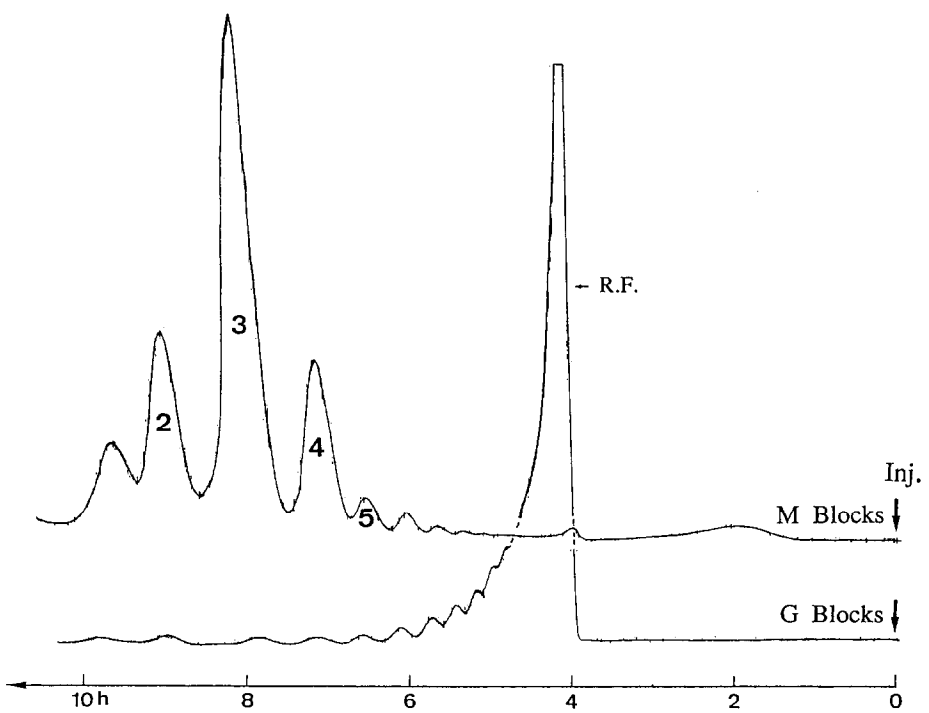

Fig. 4. Gel permeation analysis of M blocks and G blocks incubated in the presence of *Haliotis tuberculata* lyase. Bio-Gel P-6 column, 100×4.5 cm; flow rate, 90 mL min^{-1}; eluent, 50 mM $NaNO_3$. Numbers indicate the dp values of oligo-alginates and the high molecular weight material is referred to R.F., the resistant fraction.

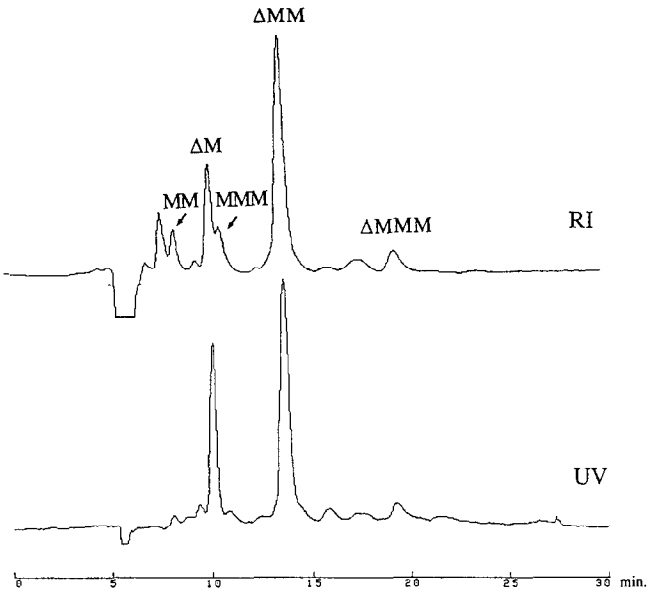

Fig. 5. High performance liquid chromatography analysis of degradation products of M blocks by *Haliotis tuberculata* lyase. Nucleosil 5 μm SB column; flow rate, 0.6 mL min^{-1}; eluent, 150 mM $NaNO_3$; on line detection with RI and UV monitors. Δ Refers to 4-deoxy-L-*erythro*-hex-4-enopyranosyluronate.

similar or longer than that of the oligomer with a dp immediately superior in the corresponding saturated series. Higher capacity factors have also been observed for unsaturated oligogalacturonates separated with a CarboPac PA1 column in high performance anion exchange chromatography. They were accounted for by the lower pKa values of the unsaturated ring, resulting from the conjugation of the double bond with the carboxyl group [32,33].

As described below, this approach is powerful enough to follow the reaction pattern of alginate lyases on pure oligomers. With mixtures of oligo-mannuronates and -guluronates, however, peak overlapping rapidly occurs for oligomers with a dp of 3 or

Table 1
Reaction products observed in the course of degradation of oligomannuronates by *H. tuberculata* alginate lyase

Substrate	MM	ΔM	MMM	ΔMM	MMMM	ΔMMM	MMMMM	ΔMMMM	MMMMMM
Reaction products					ΔMM [a]	ΔMM	ΔMMM	ΔMMM	ΔMMM
	MM	ΔM	ΔM	ΔMM	ΔM	Y	ΔMM [a]	ΔMM [a]	ΔMM [a]
			X		MM		MM [a]	ΔM [a]	MMM [a]
					X [a]		X	Y	MM
							Y		ΔM, X, Y

[a] Major components in the reaction mixture at the end of enzyme degradation. X and Y refer to unidentified carbohydrates, which we interpret as being mannuronolacton and the corresponding unsaturated monomer (Δ), respectively.

more and this HPLC procedure only allows for the rapid identification of short oligouronates. Taking into account the presence of oligomers with alternating structures in alginates, it is then obvious that this approach cannot be directly applied to the unequivocal analysis of alginate biodegradation products. However, one can take advantage of the overall trend suggested in Fig. 3 for both saturated and unsaturated oligo-alginates: for a given dp, the elution volume increases with the proportion of mannuronate residues. HPLC Analysis can therefore be combined with size-fractionation by gel permeation chromatography (GPC) and by ^{1}H NMR analysis [26] to investigate the action mechanism and specificity of alginate lyases.

High performance liquid chromatography analysis of the action pattern of Haliotis-tuberculata *alginate lyase.*—The *Haliotis tuberculata* alginate lyase was shown previously to be highly active on M-blocks and native alginate, but inactive on G-blocks [26–30]. Consistently, as shown by gel permeation chromatography on Bio-Gel P-6, only the polymannuronic blocks were depolymerized by the enzyme preparation (Fig. 4). The M-blocks digest was further analyzed by HPLC, using refractive index and UV spectrometer detectors as well as the standard calibration data of Fig. 3 for the identification of the various saturated and unsaturated oligomers (Fig. 5). At the end of the reaction, a mixture composed of a majority of low-dp unsaturated oligomannuronates was obtained, among which a trimer identified as *O*-(4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronic acid)-(1 → 4)-*O*-(β-D-mannopyranosyluronic acid)-(1 → 4)-*O*-β-D-mannopyranuronic acid (ΔMM) was accumulated. Significant proportions of saturated oligomers were also observed, that are accounted for by the low average dp (≅ 10) of the initial substrate [23].

With the aim of further understanding the cleavage pattern of *H. tuberculata* lyase, pure saturated and unsaturated oligomannuronates were subjected to enzyme hydrolysis

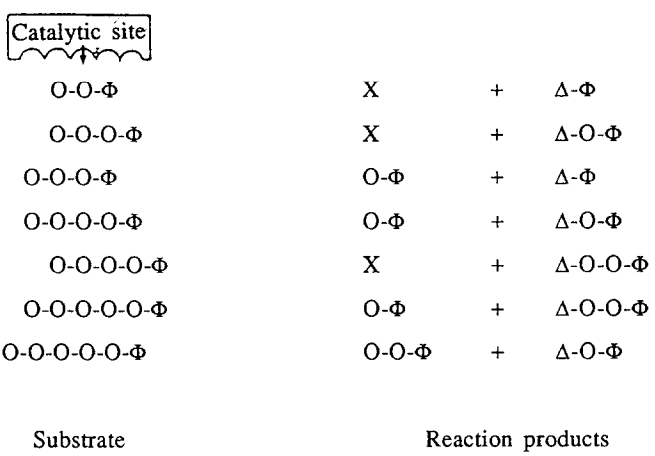

Fig. 6. Schematic representation of the recognition of oligomannuronates by the catalytic site of *Haliotis tuberculata* alginate lyase; O refers to saturated mannuronate residues, Φ to reducing ends and Δ to unsaturated non-reducing ends; X refers to an unidentified carbohydrate, which we interpret as being mannuronolacton or mannuronic acid. The arrow indicates the mannuronate residue affected by β-elimination.

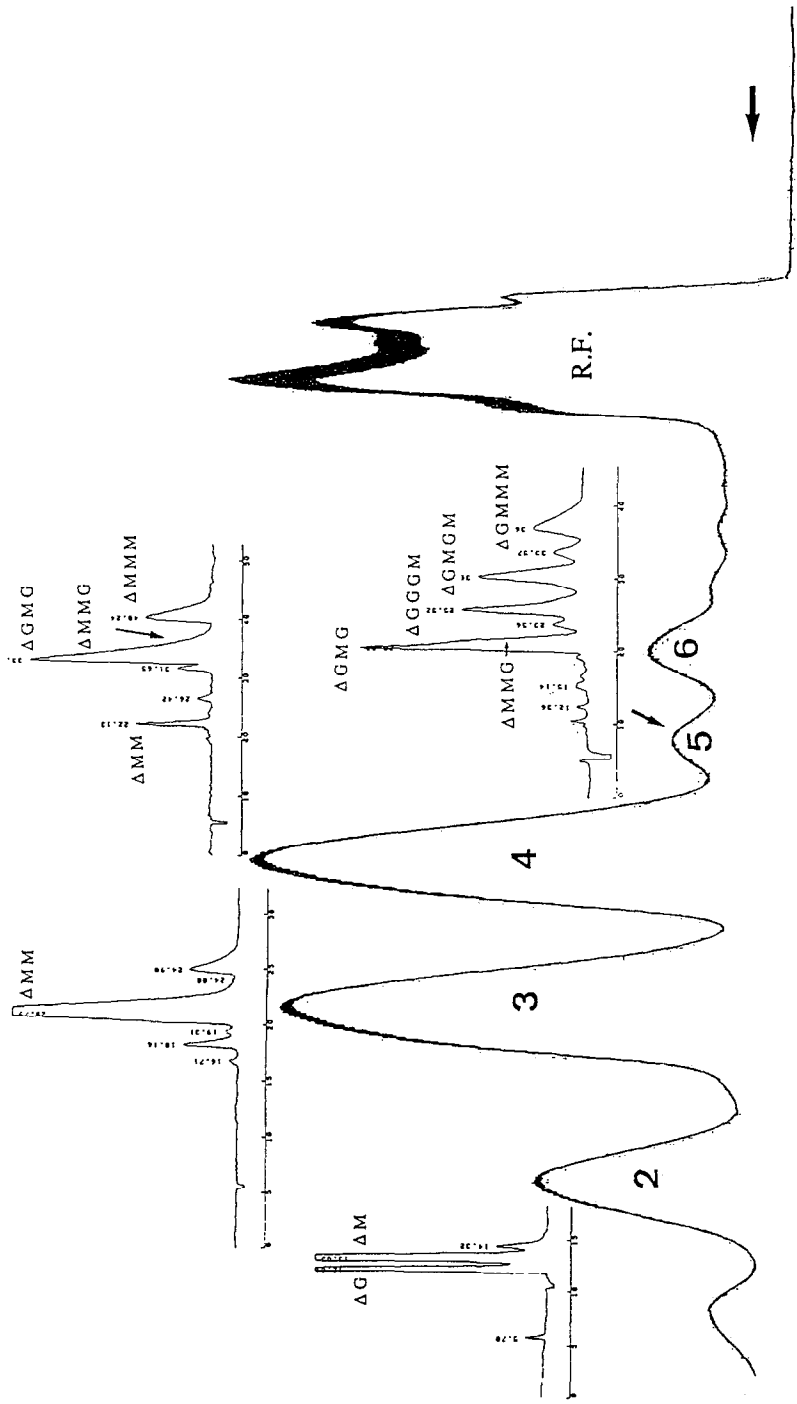

Fig. 7. Gel permeation and HPLC analysis of the degradation of *Macrocystis pyrifera* alginate by *Haliotis tuberculata* lyase. *Macrocystis pyrifera* alginate (1 mg/mL in distilled water) was incubated at room temperature overnight in the presence of 10 units of lyase. The reaction mixture was chromatographed on a Biogel P-6 column (100×4.5 cm), using 50 mM $NaNO_3$ as eluent (90 mL h^{-1}). Numbers refer to the dp values of oligo-alginates and R.F. indicates the resistant fraction. Each low molecular weight peak of the gel permeation chromatogram was further fractionated by HPLC (as shown in inserts) and analyzed by ^1H NMR.

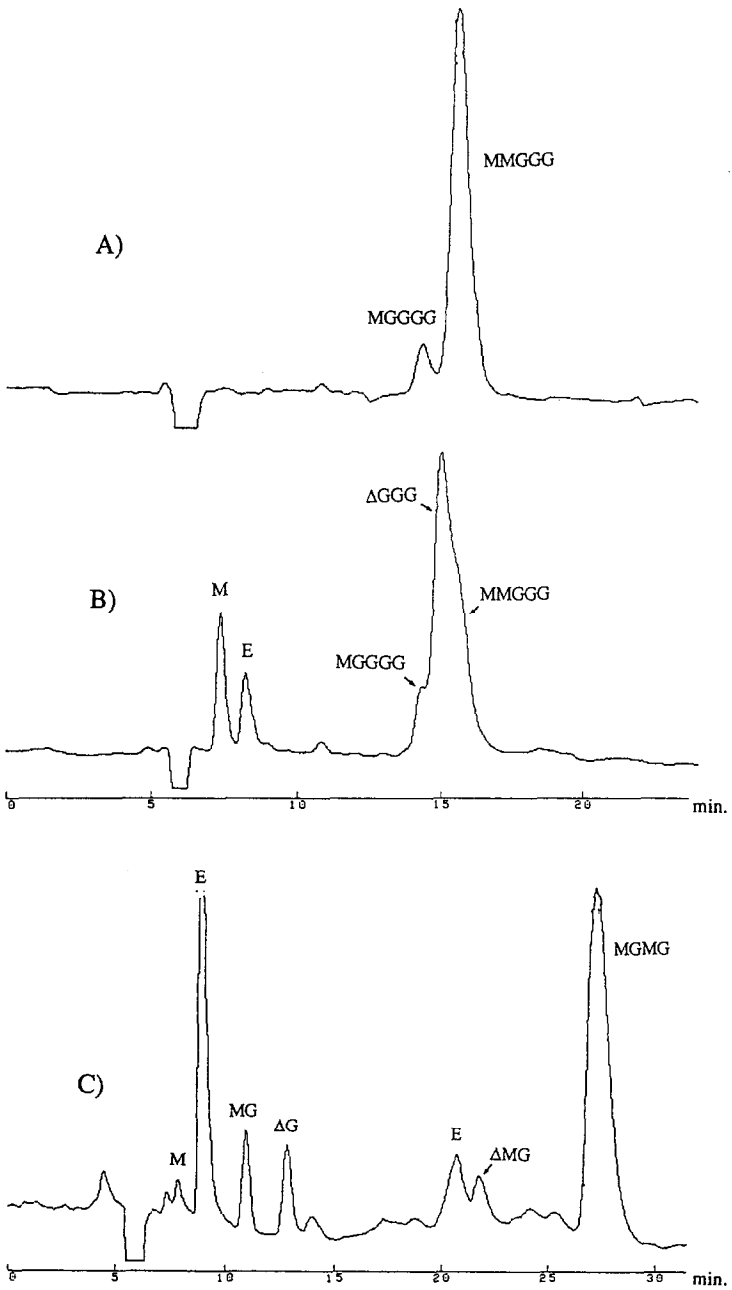

Fig. 8. High performance liquid chromatography monitoring of the hydrolysis of the pentamer MMGGG and of the tetramer MGMG by *Haliotis tuberculata* lyase. Nucleosil 5 μm SB column, RI detection; eluents were 200 mM (A, B) and 150 mM (C) $NaNO_3$ at the flow rate of 0.6 mL min^{-1}. (A) Initial MMGGG substrate; (B) reaction mixture after 30 min hydrolysis of MMGGG (ca. 5 mg/mL) with 1 lyase unit; (C) reaction mixture after 2 h hydrolysis of MGMG (ca. 5 mg/mL) with 10 lyase units. E refers to an unknown compound brought in by the enzyme preparation.

and the reaction mixtures were monitored by HPLC. The reaction products, identified during the course of the enzymatic hydrolysis, are listed in Table 1. The lyase is not capable of splitting unsaturated oligomannuronates with a dp of 3 or less, whereas the saturated trimannuronate, MMM, is cut with a low reaction rate. When longer oligomannuronates were used as substrates, the trimer ΔMM was accumulated, confirming that this entity represents an end-product of the reaction. Accumulation of unsaturated trimers as the predominant component in alginate lyase end-products appears as a consistent result, irrespective of the lyase origin and specificity [9,12,22]. From the comparison of the rate constants of disappearance, k, of purified saturated or unsaturated oligomannuronates in equimolar solutions, the pentamer MMMMM was shown to be the most readily degraded, ($kM_5/kM_4 \cong 10$; $kM_5/kM_6 \cong 2.5$). From these results, we propose that the catalytic site of *Haliotis tuberculata* lyase could best accommodate an oligomannuronate pentamer, which is cleaved into an unsaturated trimer and a saturated dimer (Fig. 6).

To check the ability of *H. tuberculata* lyase to cleave linkages other than M–M [26–30], the degradation of *Macrocystis pyrifera* alginate was monitored by both GPC and HPLC. The gel permeation chromatogram obtained after degradation of the polymer is reported in Fig. 7. Each peak corresponding to a particular dp was isolated and further analyzed by HPLC (as shown in inserts) and ^{1}H NMR spectroscopy. Interestingly, a significant proportion of the oligo-alginates generated by the lyase contained a G residue at their reducing end, a feature associated with the cleavage of G–M linkages. The question of whether M–G linkages also are attacked by the lyase was addressed by monitoring the hydrolysis of the alternating tetramer MGMG and of the pentamer MMGGG (Fig. 8). As M and ΔGGG were the main degradation products of the pentamer (Fig. 8B), the lyase is clearly highly specific for adjacent mannuronic units. In the case of the alternating tetramer, a large enzyme excess was required to observe but a limited hydrolysis, leading to the release of MG and ΔG as main products (Fig. 8C). Both results indicate that the lyase does not perform β-elimination within M–G diads with a significant reaction rate.

In conclusion, we confirm that the abalone lyase cannot be thought of as a polymannuronate lyase, since it can break G–M linkages down. In contrast, the reaction rate for the cleavage of M–G diads is very low. As it is not capable either of degrading polyguluronate blocks, this lyase apparently cannot use a guluronate unit for β-elimination, whatever its neighbour at the anomeric end of the glycosidic bond. This lyase should therefore be referred to as a mannuronate β-eliminase, indicating that the enzyme performs β-elimination on mannuronate residues only, yet from both M–M and G–M diads.

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