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Catalytic properties and specificity of a recombinant, overexpressed D-mannuronate lyase

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Abstract

Lysis of alginates and of their saturated and unsaturated fragments was monitored by ^{1}H NMR spectroscopy. $AlxM_B$ alginate lyase performs β-elimination on the mannuronic acid (M) residues. It does not cleave the guluronic acid (G) sequences, nor the M–G or the G–M diads. In consequence, it is a true mannuronate lyase. The end product of the reaction is *O*-(4-deoxy-α-L-*erythro*-hex-4-enopyranosyl-uronic acid)-(1→(4)-O-(β-D-mannopyranosyluronic acid)-(1→4)-O-β-D-mannopyranuronic acid. Viscosity measurements made during degradation of a polymannuronate alginate showed that $AlxM_B$ behaves as an endo-enzyme. HPLC analysis of the degradation products of oligomannuronates and oligoalginates suggested that the β-elimination requires the interaction of the enzyme with at least three sequential mannuronic acid residues. The catalytic site may possess 5 sub-sites and accommodate pentamers with different M/G ratio. Kinetic measurements showed that the specificity constant V_m/K_m increased with the number of mannuronic acid residues. $AlxM_B$ may be reversibly inhibited by heteropolymeric blocks in a competitive manner.

Keywords: Bacterial alginate lyase; Mannuronate lyase; $AlxM_B$

1. Introduction

Alginates are linear anionic binary copolymers of (1→4) linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues produced by marine brown algae and by a limited range of Gram-negative bacteria. It is now well established that, except for some bacterial alginates, they consist of a sequential arrangement of homopolymeric blocks of poly-β-(1→4)-D-mannuronic acid, similar blocks of poly-*a*-(1→4)- L-guluronic acid (termed M- and G-blocks, respectively) and regions in which the arrangement of the two monomers is disordered, or possibly random [1,2]. Alginates can be enzymatically degraded and the enzymes able to depolymerize alginate are almost exclusively lyases.

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Lyases catalyze the breakdown of alginate by a β-elimination mechanism, causing formation of a double bond between C-4 and C-5, elimination of the 4-O-glycosyl substituent and production of 4-deoxy-L-*erythro*-hex-4-ene pyranosyluronate at the non reducing end of the resulting oligosaccharides [3,4].

Among the many alginate-depolymerizing enzymes which have been obtained from marine algae, mollusks and bacterial sources, a few of them have a known primary structure [5–12].

Four of them have been overproduced [10–14]. As four types of linkages have to be considered, the problem of defining specificity is complex and difficult, and the specificity of the alginate lyases is generally described in terms of preferential degradation of block structures enriched in either mannuronate or guluronate residues. Recently, HPLC analysis of homologous series of saturated and unsaturated oligo-mannuronates and -guluronates and their characterization by ^{1}H- and ^{13}C-nmr spectroscopy were reported [15,16]. Taking advantage of the availability of these oligoalginates with well-defined structures and of the powerful tools developed, the true substrate specificity and degradation mechanism of the overproduced bacterial mannuronate lyase $AlxM_B$ [14], has been investigated and is reported in this paper.

2. Experimental

Alginate lyase.—The recombinant enzyme $AlxM_B$ (85 U/mg protein) was obtained as described by Malissard et al. [14].

Substrates.—Polymannuronate bacterial alginate (A-12) from a *Pseudomonas aeruginosa* clinical strain was deacetylated (see the following paper). Low-viscosity sodium alginate from *Macrocystis pyrifera* (60 % mannuronate) was purchased from Sigma. G-blocks (with about 5 % of M residues) were prepared according to Haug et al. [17]. M-blocks and MG-blocks were prepared from bacterial alginates as reported by Heyraud et al. [18]. Saturated and unsaturated oligomers were fractionated and identified as described by Heyraud et al. [15].

Enzyme assays.—Buffer A was 75 mM sodium phosphate pH 7.5 / 450 mM NaCl. Enzyme solutions (400μl) containing up to 0.3 % substrate were incubated for 1 to 5 min at 37 °C. The unsaturated non-reducing groups produced by β-elimination were measured using 3-deoxy-D-*manno*-octulosonic acid (up to 50 nmol) as a standard. The conditions described by Karkhanis et al. [19], were modified according to Malissard et al. [14]. One unit of enzyme activity produced 1 μmol of non-reducing, unsaturated, terminal group per min. Kinetic parameters were calculated from a linear curve-fitting program (*K*cat 1.31, Macintosh).

Specificity profile of $AlxM_B$.—Enzyme (0.085 U) was added to various alginate oligomers (10 M) in buffer A (500 μL). After 15 min at 37 °C, the hydrolysis products were analyzed by HPLC on an anion exchange column.

NMR spectroscopy.—NMR analysis was performed at 85 °C, using an AC-300 Bruker Fourier transformation spectrometer with a 5 mm ^{1}H, ^{13}C dual probe. ^{1}H NMR spectra were obtained using a spectral width of 3000 Hz, a 16 K data-block size and a pulse duration of 8 μs; 16 scans with AQ = 2.73 s were accumulated. The H_2O signal was presaturated using the standard Bruker Presat sequence, with a delay of 3 s and a decoupler power of 20 dB at low range.

High performance liquid chromatography.—Anion-exchange liquid chromatography was performed on a SB Nucleosil 5 μm prepacked column (250×4.6 mm) from Interchim (France). Chromatography was run isocratically at room temperature with a 0.2 M $NaNO_3$ solution at a flow rate of 0.6 mL min^{-1}. Detection was performed on line with a R-410 Waters refractometric detector and a Model 481 UV LC spectrophotometer operating at 254 nm.

Viscosity measurements.—They were obtained at 37 °C, using an Ubbelhode viscometer. At 10 mL of a 0.5 mg mL^{-1} deacetylated bacterial alginate A-12, enzyme (3.8 10^{-4} U) was added, and the variation of viscosity was monitored in continuity.

3. Results and Discussion

Substrate specificity of $AlxM_B$.—Degradation by the lyase of G-, M- and MG-blocks and also of a commercial *Macrocystis pyrifera* alginate was monitored by ^{1}H NMR . The M-blocks (Fig. 1c) were degraded into a mixture of saturated and unsaturated oligouronides. Integration of the appropriate signals of protons in the region 4.7–5.7 ppm, indicated an average dp of 3 for the end-product: *O*-(4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronic acid)-(1→4)-O-(β-D-mannopyranosyl-

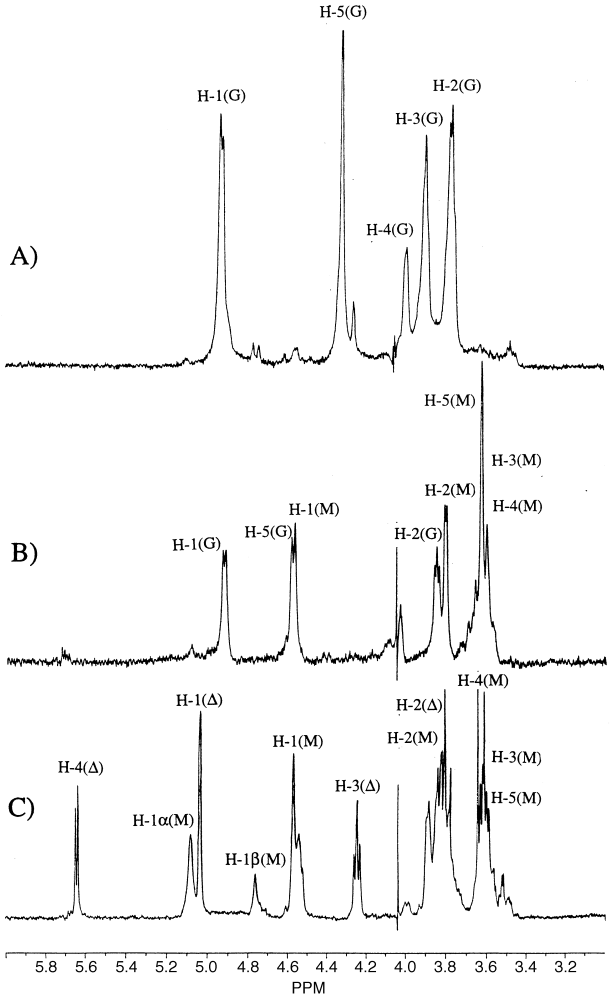

Fig. 1. Proton NMR (300 MHz, 85 °C) spectra of 0.5 mL solutions (10 mg mL^{-1} in D_2O at pD 7) of (A) a polyguluronate block (G-block), (B) an alternate block (MG-block) and (C) a polymannuronate block (M-block) after 15 min incubation at 37 °C in the presence of 1.1 U of $AlxM_B$. α and β refer to the signals of anomeric protons; Δ refers to the unsaturated uronic acid at the non-reducing end.

uronic acid)-(1→4)-O-β-D-mannopyranuronic acid (ΔMM). This result was based on the complete assignment of various saturated or unsaturated oligouronates, as indicated by Heyraud et al. [16]. The spectra of G -blocks (Fig. 1a) and MG-blocks (Fig. 1b) remaining unchanged, the enzyme is clearly acting as a mannuronate lyase, a result consistent with previous reports [6,14].

In contrast to the *Haliotis tuberculata* alginate lyase, which performs β-elimination on mannuronate residues from both the M–M and G–M diads of alginates [15,16], the recombinant enzyme $AlxM_B$ is only active on M–M diads. This is clearly corroborated by the ^{1}H NMR spectrum of the products obtained from the *Macrocystis pyrifera* alginate (Fig. 2). Only a single peak at 4.75–4.80 ppm, corresponding to the β-anomer of a mannuronate unit located at the reducing-end of an oligomer formed by the cleavage of a M–M diad, was observed. A mannuronate or a guluronate lyase activity on G–M or G–G diads, respectively, should have lead to the appearance of a doublet at 4.70–4.75 ppm.

Process of $AlxM_B$ activity.—The degradation of the deacetylated polymannuronate A-12 was directly followed in a viscometer at 37 °C . Assuming random cleavage, the molecular weight, M, varies with time, t, as:

$$1/M = 1/M_0 + kt \quad (1)$$

in which, M_0 is the initial molecular weight and k is a rate constant [20].

By writing: $[\eta] = KM^a$, relation (1) becomes:

$$1/[\eta]^{1a} = 1/[\eta]_0^{1/a} + k_1 t$$

$$\left[[\eta]_0/[\eta]\right]^{1/a} = 1 + k_2 t \quad (2)$$

in which $[\eta]_0$ is the initial intrinsic viscosity and $[\eta]$ the intrinsic viscosity after a time of hydrolysis t.

For low polymer concentrations, one may assume that:

$$\left[[\eta]_0/[\eta]\right]^{1/a} \approx [\eta_{0red}/\eta_{red}]^{1/a} = \text{const.} + k_3 t \quad (3)$$

where η_{0red} and η_{red} are the reduced viscosities, $\eta_{red} = (\eta - \eta_s)/\eta_s.c$, c being the polymer concentration and η_s and η the viscosity of solvent and alginate solution, respectively]. The reduced viscosities are calculated from the viscosity measurements, and the coefficient, a, related to the rigidity of the molecule, is set to 0.85, assuming a semi-rigid behavior for the alginate chain [21,22].

The decrease in viscosity with time due to the enzymatic degradation of the polymannuronate sample is shown in Fig. 3. The linear dependence observed indicates a random mechanism of hydrolysis by an endo-enzyme. No inhibition phenomenon could be detected and doubling the amount of enzyme after 2 h led to an increase in the degradation rate by a factor 2.

Kinetic parameters of $AlxM_B$.—The kinetic parameters were estimated in buffer A after incubation of $AlxM_B$ (0.01 U) for 3 min at 37 °C with

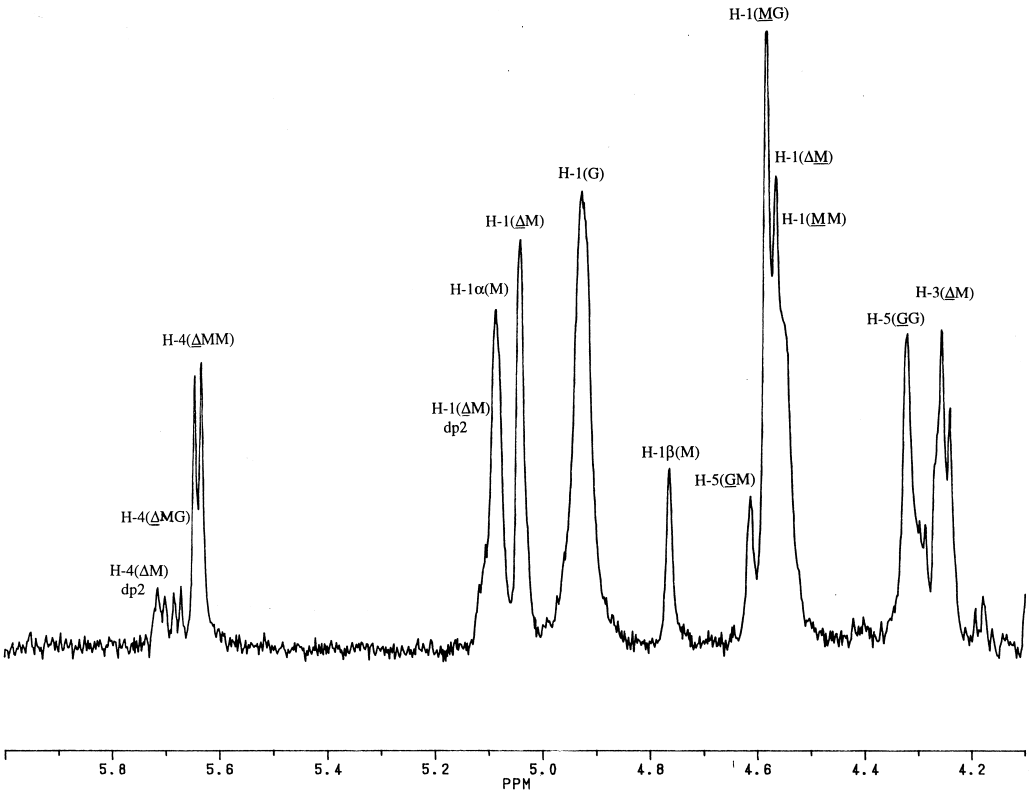

Fig. 2. Signal assignment of the most relevant protons in the ^{1}H NMR spectrum of the end products of a 0.5 mL solution of *Macrocystis pyrifera* alginate (10 mg mL^{-1}) after 15 min incubation at 37 °C with 1.1 U of AlxM_B alginate lyase. The spectrum was recorded at 300 MHz and 85 °C. α and β refer to the signals of anomeric protons; Δ refers to the unsaturated uronic acid at the non-reducing end; underlining refers to the considered unit.

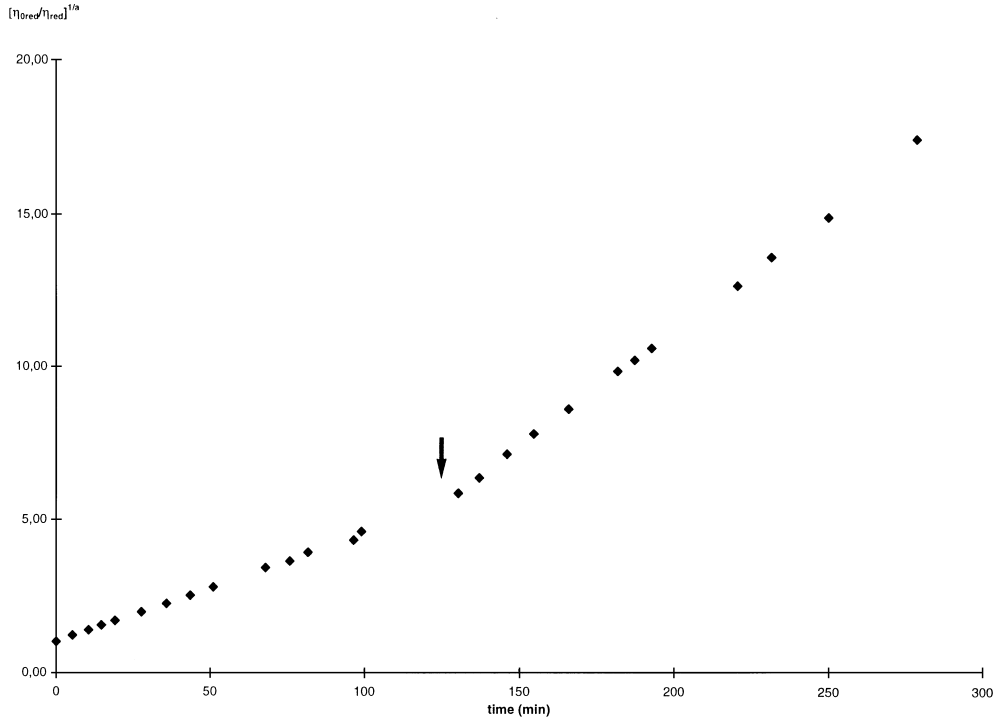

Fig. 3. Variation of the ration $[\eta_{0red}/\eta_{red}]^{1/a}$ as a function of the time at 37 °C for a 10 mL solution of a polymannuronate alginate (0.5 mg mL^{-1}) in the presence of 3.8 10^{-4} U of AlxM_B alginate lyase. Arrow indicates the addition of a new amount of enzyme (3.8 10^{-4} U).

the following substrates: 0.02–0.75 mM M-blocks, 0.09–2.98 mM M5 and 0.63–20 μM *M. pyrifera* alginate. The K_m and apparent V_m values are listed in Table 1. It must be stressed, from these values, that the specificity constant V_m/K_m strongly increases with the molecular size of the substrates indicating a higher specificity of the enzyme for long chain alginates. This result was further investigated with 8 mU of enzyme and 12.5 and 25 μM oligomannuronates (M_4, M_5, M_7) in buffer A (400 μL) for 1 min at 37 °C. In these conditions, the initial velocities were increasing with molecular size too, rates for M_4 and M_5 being 35 and 80%, respectively, of the value for M7.

Specificity profile of AlxM$_B$.—Various alginate oligomers were submitted to the action of AlxM_B. The degradation products were analyzed by HPLC. The results with oligomannuronates and oligoalginates are reported in Tables 2 and 3, respectively. They suggest an active site with 5 subsites. AlxM_B cleaves the pentamer MMMMM into the saturated dimer MM, and the unsaturated trimer ΔMM. Analysis of the fragments released by the pentamers with different M/G ratios (Table 3) shows that at least three sequential mannuronic acid residues must be involved in the interaction. This explains the ineffectiveness of the enzyme on the MG-blocks observed in Fig. 1(b).

Reaction rate for M-blocks as a function of AlxM$_B$ concentration.—The formation of unsaturated non-reducing end-groups, at a constant substrate concentration of M-blocks (3 mg mL^{-1} in buffer A), was studied as a function of time with increasing concentrations of AlxM_B (Fig. 4).In each case, the same and final maximum value of degraded products (up to 8000 nmol) was obtained

Table 1
Kinetic parameters of AlxM_B

Substrate	Concentration μM	K_m μM	V_m μmol min^{-1} mg^{-1}	V_m/K_m L min^{-1} mg^{-1}
M. pyrifera alginate	0.6–20	11	141	12.8
M-blocks	20–750	60	139	2.5
M_5	90–2980	180	103	0.6

Incubations in buffer A (400 μL) were performed at 37 °C for 3 min with 0.01 U of AlxM_B. The values of unsaturated, non-reducing end-groups were estimated and the kinetic parameters were determined using the Kcat 1.31 software; M_5 is the pentamannuronate (dp 5).

Table 2
Sites of β-elimination with oligomannuronates as substrates

	M_2	ΔM	M_3	ΔM_2	M_4	ΔM_3	M_5	ΔM_4	M_6	M_7
Final products	n.d	n.d	n.d	n.d	M_1 ΔM_2	ΔM	M_1 M_2[a] ΔM_2[a] ΔM_3	ΔM ΔM_2	M_1 M_2 M_3[a] ΔM ΔM_2[a]	M_1,M_3 ΔM ΔM_2[a]
Major site of β-elimination[b]					M-MMM		MM-MMM		MMM-MMM	M-MMM-MMM

Δ refers to the 4-deoxy-L-*erythro*-hex-4-enopyranosyluronate located at the non-reducing end, indices indicate the number of saturated uronic residues; [a]major products; [b]hyphens indicate the site β-elimination.

Table 3
Sites of β-elimination with oligoalginates as substrates

	MMMMM	MGGGG	MMGGG	MMMGG	MMGGM	MMMMG
Final products	M_1 M_2[a] ΔM_2[a] ΔM_3	n.d	n.d	M_1 ΔMGG	n.d	M_1 M_2 ΔMG[a] ΔMMG
Major site of β-elimination[b]	MM-MMM			M-MMGG		MM-MMG

Δ refers to the 4-deoxy-L-*erythro*-hex-4-enopyranosyluronate located at the non-reducing end, indices indicate the number of saturated uronic residues; n.d. no degradation; [a]major products; [b]hyphens indicate the site of β-elimination.

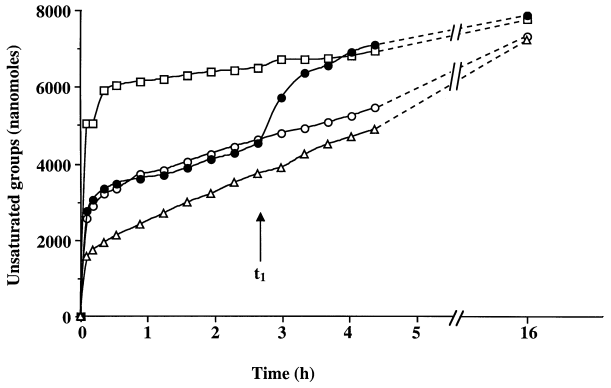

Fig. 4. Degradation of M-blocks by $AlxM_B$ as a function of time. M-blocks (0.75 mM) were treated with 0.06(Δ), 0.12(0), 0.12 + 1.14(●), and 1.14([]), U of $AlxM_B$. Assays (2 mL) were incubated at 37 °C, samples (20 μL) were removed at intervals and unsaturated groups were quantified. At time t_1 (155 min) enzyme (2.3 U) was added in assay (●).

after 16 h of incubation but the reaction rate was increased by addition of enzyme after 155 min (time t_1, Fig. 4).

Reaction rate for alginate as a function of AlxM$_B$ concentration.—Degradation of *M. pyrifera* alginate (3 mg mL^{-1} in buffer A) was followed with amounts of $AlxM_B$ ranging from 0.076 to 2.3 U. Fig. 5 shows that the yields of unsaturated non-reducing end groups quickly rose to constant values that depended upon the initial concentration of enzyme. Addition of more enzyme (time t_1) led to an increase in yield, and, addition of more substrate (time t_2) when the maximum level was reached again led to the formation of more product. Therefore, AlxM*B* is subject to reversible product inhibition upon degradation of *M. pyrifera*.

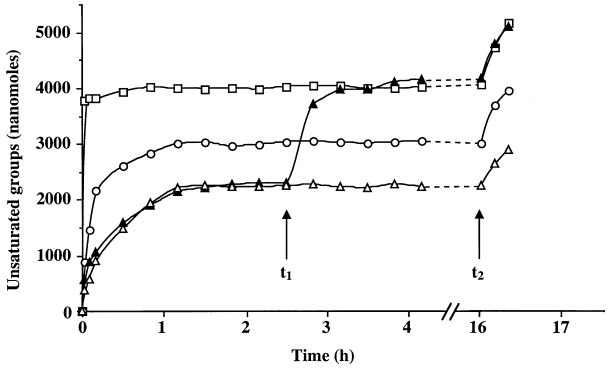

Fig. 5. Degradation of *Macrocystis pyrifera* alginate as a function of $AlxM_B$ concentration. Alginate (6 mg) was treated with 0.08 (Δ), 0.16 (○), 2.3 ([]), and 0.08 + 2.3 (▲) U of $AlxM_B$. Assays (2 mL) were quantified as described in legend for Fig. 3. At time t_1 (150 min) enzyme (2.3 U) was added in assay (▲) . At time t_2 (16 h) alginate (6 mg) was added in all samples.

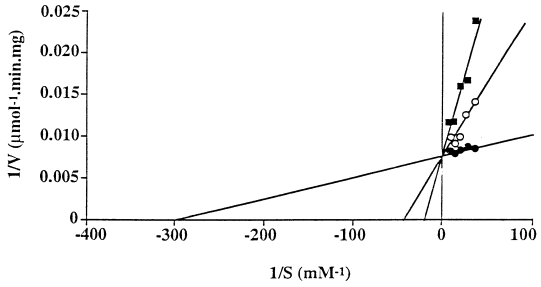

Fig. 6. Inhibition of $AlxM_B$ activity. M-blocks (0.02 to 0.15 mM in buffer A) were treated with $AlxM_B$ (0.01 U) at 37 °C for 3 min. Plots of $1/V$ versus $1/[s]$ are presented in the presence of the following amounts of unsaturated oligomers from *M. pyrifera* alginate: 0(●), 88(○), 143(■) nmoles.

Contrary to the previous results of Larsen et al. [22] with the *Haliotis tuberculata* alginate lyase, this behaviour was not observed with M-blocks (Fig. 4) or polymannuronate. *Macrocystis pyrifera* alginate was submitted to a complete degradation, with multiple addition of $AlxM_B$ and the mixture of the resulting unsaturated products was collected. With the M-blocks as substrate, the kinetics of inhibition of $AlxM_B$ by these unsaturated products was studied (Fig. 6). An inhibition was observed. It was competitive and could be related to oligosaccharides with MG sequences.

In conclusion, $AlxM_B$ can be listed as a pure mannuronate lyase. This specificity should be very useful for the enzymatic preparation of uronate blocks with a well-defined structure. The latter will be needed to define the specificities of other alginate lyases. Moreover, the activity of the two mannuronate lyases $AlxM_B$ and *Haliotis tuberculata* alginate lyase could be compared on various alginates with the aim of refining the structure and properties of such substrates. Both enzymes are able to perform β-elimination on the sole mannuronic acid residues but the latter one works independently of the immediate neighbors. This should provide more information about the length and organization of the alternated zones of uronic acids in alginates.

References

[1] A. Haug, B. Larsen, and O. Smidsrøød, *Acta Chem. Scand.*, 20 (1996) 183–190.
[2] A. Haug, B. Larsen, O. Smidsrøød, and T. Painter, *Acta Chem. Scand.*, 23 (1996) 2955–2962.
[3] P. Gacesa, *Int. J. Biochem.*, 24 (1992) 545–552.
[4] K. Murata, T. Inose, T. Hisano, S. Abe, Y. Yonemoto, T. Yamashita, M. Takagi, K. Sakaguchi,

A. Kimura, and T. Imanaka, *J. Ferment. Bioeng.*, 76 (1993) 427–437.
[5] Y. Yonemoto, H. Tanaka, T. Hisano, K. Sakaguchi, S. Abe, T. Yamashita, A. Kimura, and K. Murata, *J. Ferment. Bioeng.*, 75 (1993) 336–342.
[6] M. Malissard, C. Duez, M. Guinand, M.J. Vacheron, G. Michel, N. Marty, B. Joris, I. Thamm, and J.M. Ghuysen, *FEMS Microbiol. Lett.*, 110 (1993) 101-106.
[7] H. Maki, A. Mori, K. Fujiyama, S. Kinoshita, and T. Yoshida, *J. Gen. Microbiol.*, 139 (1993) 987–993.
[8] N.L. Schiller, S.R. Monday, C.M. Boyd, N.T. Keen, and D.E. Ohman, *J. Bacteriol.*, 175 (1993) 4780–4789.
[9] A. Boyd, M. Ghosh, T.B. May, D. Shinabarger, R. Keogh, and A.M. Chakrabarty, *Gene*, 131 (1993) 1–8.
[10] A.J. Baron, T.Y. Wong, S.J. Hicks, P. Gacesa, D. Willcock, and M.J. McPherson, *Gene*, 143 (1994) 61–66.
[11] F. Chavagnat, C. Duez, M. Guinand, P. Potin, T. Babeyron, B. Henrissat, J. Wallach, and J.M. Ghuysen, *Biochem. J.*, 319 (1996) 575–583.
[12] T. Muramatsu, K. Komori, N. Sakurai, K. Yamada, Y. Awasaki, K. Fukuda, and T. Oda, *J. Prot. Chem.*, 15 (1996) 709–719.
[13] T. Hisano, M. Nishimura, T. Yamashita, K. Sakaguchi, M Takagi, T. Imanaka, A. Kimura, and K. Murata, *J. Ferment. Bioeng.*, 78 (1994) 79-83.
[14] M. Malissard, F. Chavagnat, C. Duez, M.J. Vacheron, M. Guinand, G. Michel, and J.M. Ghuysen, *FEMS Microbiol. Lett.*, 126 (1995) 105–112.
[15] A. Heyraud, Ph. Colin-Morel, S. Girond, C. Richard, and B. Kloareg, *Carbohydr. Res.*, 291 (1996) 115–126.
[16] A. Heyraud, C. Gey, C. Leonard, C. Rochas, S. Girond, and B. Kloareg, *Carbohydr. Res.*, 289 (1996) 11–23.
[17] A. Haug, B. Larsen, and O. Smidsrød, *Acta Chem. Scand.*, 21 (1967) 691–704.
[18] A. Heyraud, P. Colin-Morel, C. Gey, F. Chavagnat, M. Guinand, and J. Wallach, *Carbohydr. Res.*, 308 (1998) 417–422.
[19] Y.D. Karkhanis, J.Y. Zeltner, J.J. Jackson, and D.J. Carlo. *Anal. Biochem.*, 85 (1978) 595–601.
[20] M. Rinaudo and M. Milas, *Int. J. Biol. Macromol.*, 2 (1980) 45–48.
[21] O. Smidsrød and A. Haug, *Acta Chem. Scand.*, 22 (1968) 797–810.
[22] W. Mackie, R. Noy, and D.B. Sellen, *Biopolymers*, 19 (1980) 1839–1860.
[23] B. Larsen, K. Koøen, and K. Oøstgaard, *Hydrobiologia*, 260 (1993) 557–561.