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Novel alginate lyases from marine bacterium *Alteromonas* sp. strain H-4

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Abstract

A bacterium *Alteromonas* sp. strain H-4 isolated from *Laminaria* fronds produced extra- and intra-cellular alginate lyases and utilized alginate as its sole carbon source. An extracellular alginate lyase was purified from the culture supernatant of the strain and its substrate specificity was characterized. The estimated molecular mass of the enzyme was 32 kDa and the isoelectric point was 4.7. Both polyM and polyG block degrading activities were observed using the substrate-containing gel overlay technique after isoelectric focusing of the enzyme. By analyzing the reaction products from the polyM block, polyG block, MG random block and intact alginate, three major peaks containing unsaturated tri-uronide through octa-uronide were detected for each substrate. The results indicate that the enzyme of *Alteromonas* sp. H-4 can degrade both polyM and polyG blocks with a K_m in mg/mL 20-times higher for the polyM block. © 1997 Elsevier Science Ltd.

Keywords: Alginate lyase; Substrate specificity; *Alteromonas*; Marine bacterium

1. Introduction

Alginate lyase activity has been detected from a wide variety of sources, including marine molluscs, bacteria, fungi and marine brown algae, and some of these lyases have been isolated and characterized [1,2]. Alginate is comprised of (1 → 4)-linked β-D-mannuronate (M) and α-L-guluronate (G), and these uronic acids are arranged in block structures which may be homopolymeric (polyM block and polyG block) or heteropolymeric (random sequence comprised with MG random block) [3]. Alginate lyases have been tentatively classified into two types based on their substrate specificity, defined as the preference for either polyM block or polyG block [1,2]. The alginate lyases found in *Haliotis* [4,5], *Photobacterium* sp. [6] and *Pseudomonas aeruginosa* [7] are representative of polyM lyase, and those found in *Klebsiella aerogenes* [5,8,9] and *Pseudomonas alginovora* [10] are representative of polyG lyase. However, since the uronic acid at the non-reducing end after lyase action is unsaturated, it is not possible to decide whether the uronic acid at the non-reducing end was originally M or G. As a result, there has been no progress in the detailed analysis of the substrate specificity of alginate lyases. Only Haugen et al. [5] have conducted detailed analysis of the depolymerized sequence in an alginate molecule by alginate lyases using kinetic measurement and end-group analysis of the reaction product, and they

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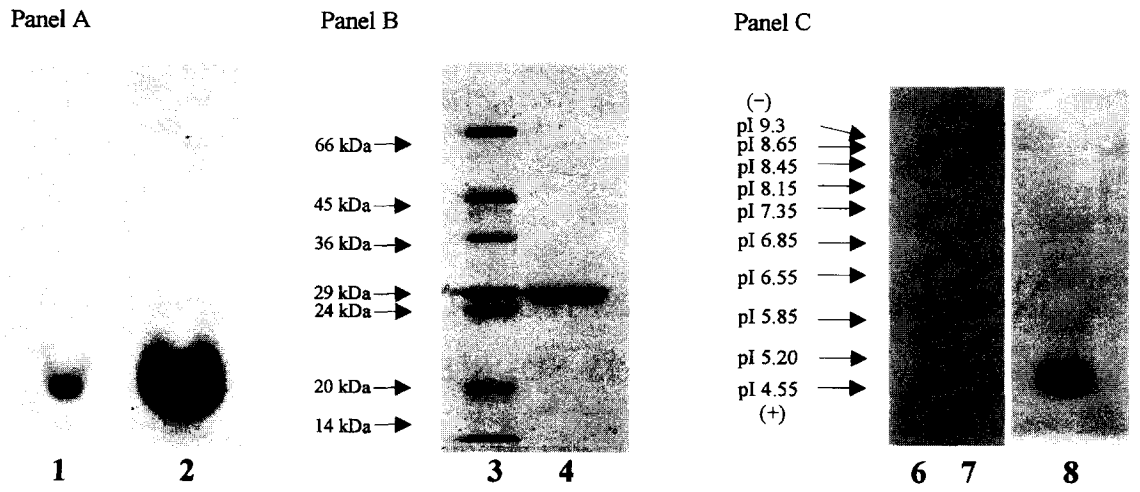

Fig. 1. Homogeneity of the purified extracellular alginate lyase of *Alteromonas* sp. H-4. (Panel A) Native-PAGE (7% gel) of the purified alginate degrading enzyme. Lane 1, the alginate lyase (3.4 μg) stained with CBB R250; lane 2, activity staining with gel-overlay containing alginate. (Panel B) SDS-PAGE (10% gel) of the purified enzyme. Lane 3, markers [albumin (bovine): 66 kDa, ovalbumin: 45 kDa, glyceraldehyde-3-phosphate dehydrogenase: 36 kDa, carbonic anhydrase: 29 kDa, trypsinogen: 24 kDa, trypsin inhibitor: 20.1 kDa, α-lactalbumin: 14.2 kDa]; lane 4, the alginate lyase (30 μg) stained with CBB R250. (Panel C) Isoelectric focusing of the enzyme. Lane 6, marker proteins [trypsinogen: p*I* 9.30, lentil lectin-basic band: p*I* 8.65, lentil lectin-middle band: p*I* 8.45, lentil lectin-acidic band: p*I* 8.15, horse myoglobin-basic band: p*I* 7.35, horse myoglobin-acidic band: p*I* 6.85, human carbonic anhydrase B: p*I* 6.55, bovine carbonic anhydrase B: p*I* 5.85, β-lactoglobulin A: p*I* 5.20, soybean trypsin inhibitor p*I* 4.55]; lane 7, the alginate lyase (0.15 μg) stained with CBB R250; lane 8, activity staining with gel-overlay containing alginate.

pointed out the direction for future investigations of the substrate specificity of alginate lyase. Compared to pectinolytic enzymes, which have been classified into several types, including one type of esterase, six types of polygalacturonases and four types of lyases [11], the enzymological details of alginolytic enzymes are poorly known. Although all alginolytic enzymes studied to date have been classified as lyases belonged to EC4.2.2.3, it is possible that other kinds of alginolytic enzymes, for example hydrolases or a unique lyase capable of degrading only oligosaccharides or with new types of substrate specificity, may be discovered.

Our laboratory has conducted ecological and physiological studies of marine alginolytic bacteria [12,13], in which we used alginolytic enzymes and genes encoding alginolytic enzymes as molecular markers. For the alginolytic isolates, an extracellular alginate lyase of the marine bacterium *Alteromonas* sp. H-4 was purified and characterized [14]. It was found that the *Alteromonas* alginate lyase was stable and very active for alginate degradation in the marine environment [14], and effective for isolating protoplasts of *Laminaria japonica* than abalone alginase [15,16]. It is possible that the mode of depolymerization of alginate by the *Alteromonas* enzyme is different from abalone enzyme, which is known to be a polyM specific lyase [4,5]. We report here on the

Table 1
Comparison of N-terminal amino acid sequence among known alginate degrading enzymes from bacteria

Origin	Sequenced from	N-terminal amino acid sequence	Reference
Alteromonas sp. H-4	protein	GDTGSGSGIASNITNGSIFDLEGNNPHPLV	In this study
Pseudomonas sp. OS-ALG-9	gene	EKTYTISSAAELSQLNLMPGDKVIMKSGEW	[18]
Pseudomonas aeruginosa	gene	ADLVPPPGYYAAVGERKGSAGSXPAVPPPY	[20]
Vibrio SFFB 080483 A	protein	DSAPYDXXXXXXXXDXXK	[16]
Vibrio ATCC 433367 20 kDa	gene	GVEFSNP	[19]
Vibrio ATCC 433367 10 kDa	gene	KDKEMXXADV	[19]
Klebsiella pneumoniae subsp. *aerogenes*	gene and protein	AVPAPGDKFELSGWSLSVPVDSDNDGKADQ	[7]

substrate specificity and reaction products of the extra-cellular alginate lyase of *Alteromonas* sp. strain H-4, and discuss the mode of action of the enzyme.

2. Results

Homogeneity of the extra-cellular enzyme purified from the culture supernatant of *Alteromonas* sp. strain H-4 was confirmed by electrophoresis and analysis of the *N*-terminal amino acid sequence. The purified enzyme was observed to be a homogenous, single band in native-PAGE, SDS-PAGE and IEF analysis (Fig. 1, Panel A-lane 1, B-lane 4 and C-lane 7, respectively). The single protein band detected in native-PAGE and IEF analysis showed alginate-degrading activity using the Alg-Na-containing gel overlay technique (Fig. 1 panel A-lane 2 and panel C-lane 8). The estimated molecular mass and isoelectric point of the purified extracellular alginate lyase were 32 kDa and 4.7, respectively. The sequence from Edman degradation was GDTGSGSGIASNIT-NGSIFDLEGNNPHPLV. Table 1 shows comparison

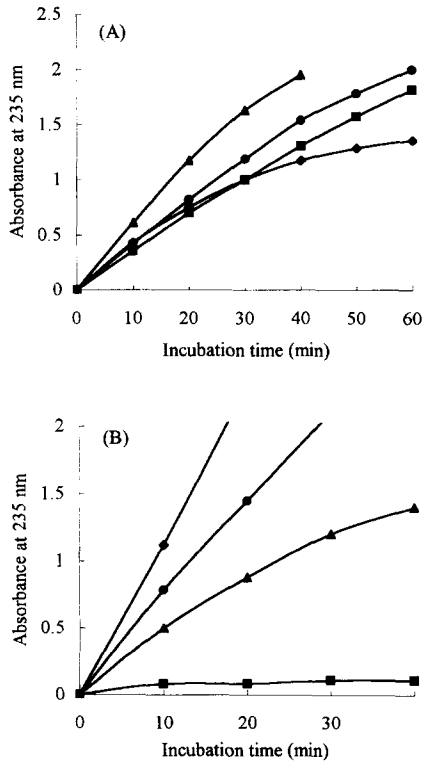

Fig. 2. Substrate specificity of the purified extracellular alginate lyase of *Alteromonas* sp. strain H-4 (A) and abalone alginate lyase (B). Alginate lyase activity at 30 °C was measured for the following substrates; sodium alginate (●), polyM block (◆), polyG block (■) and MG random block (▲) by monitoring the absorbance of reaction mixture at 235 nm.

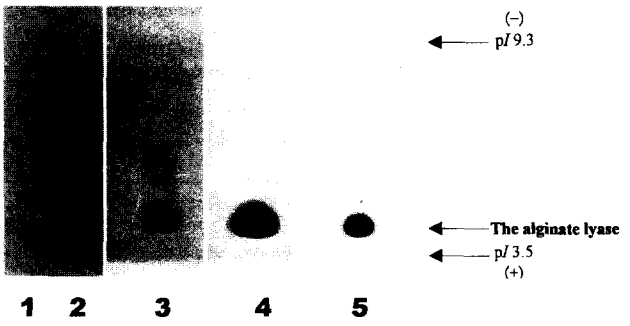

Fig. 3. Activity staining profiles of the extracellular alginate lyase of *Alteromonas* sp. H-4 for polyM block and polyG block after isoelectric focusing gel electrophoresis. Lane 1, marker proteins (see Fig. 2, panel C); lane 2, the alginate lyase (0.15 μg) stained with CBB R250; lane 3, activity staining with gel-overlay containing Alg-Na; lane 4, activity staining with gel overlay containing polyM; lane 5, activity staining with gel overlay containing polyG.

among known *N*-terminal amino acid residues of alginate lyases [6,17–20]. No alginate lyases with completely corresponding *N*-terminal amino acid has been observed.

Fig. 2 shows the alginate lyase activities of the purified extracellular enzyme of the strain and abalone acetone powder for the polyM block, polyG block, MG random block and Alg-Na. The purified enzyme from the bacterium showed lyase activity for all substrates (Fig. 2A). The initial rate of degradation seemed to be similar for all substrates. Alginate lyase activity of the abalone acetone powder was observed for the polyM block, MG random block and Alg-Na, but not for the polyG block (Fig. 2B). The lyase activity of the abalone enzyme for polyM was found to be higher than any other substrates.

The bacterial enzyme degradation for polyM block and polyG block on gel overlays (Fig. 3, lane 4 and 5) were detected at the same position on the Alg-Na-containing gel overlays after IEF (Fig. 3, lane 3). The activities corresponded to the protein band detected on the IEF gel (Fig. 3, lane 2). Two polyM block-degrading proteins with p*I* 9.20 and p*I* 6.75 were detected in abalone enzymes, but these activities did not appear in the polyG block (data not shown).

Table 2 shows the kinetic data. The K_m constants of the enzyme for the polyM block, polyG block, MG random block and Alg-Na were 1431 mg/mL, 66 mg/mL, 165 mg/mL and 229 mg/mL, respectively, at 30 °C in a 0.1 M Tris-HCl buffer (pH 7.5) based on a Lineweaver–Burke plot. The K_m value for polyM block was 20 times higher than that for polyG block. From the ratio of the selectivity coefficients (V_{max}/K_m), the efficiency for the polyG block was

Table 2
Kinetic constants of the alginate lyase from *Alteromonas* sp. strain H-4

Substrate	K_m (μg/mL)		V_{max} (O.D. 235/min/μg protein)		V_{max}/K_m	
	0.1 M Tris	$+Mg^{2+}$ [a]	0.1 M Tris	$+Mg^{2+}$ [a]	0.1 M Tris	$+Mg^{2+}$ [a]
Alg-Na	229	18	0.021	0.036	9.2×10^{-5}	2.0×10^{-3}
PolyM	1431	227	0.042	0.062	2.9×10^{-5}	2.7×10^{-4}
PolyG	66	16	0.016	0.036	2.4×10^{-4}	2.2×10^{-3}
MG random	165	14	0.029	0.032	1.8×10^{-4}	2.3×10^{-3}

[a] The reaction mixture based on 0.1 M Tris-HCl buffer (pH 7.5) containing 50 mM $MgCl_2$.

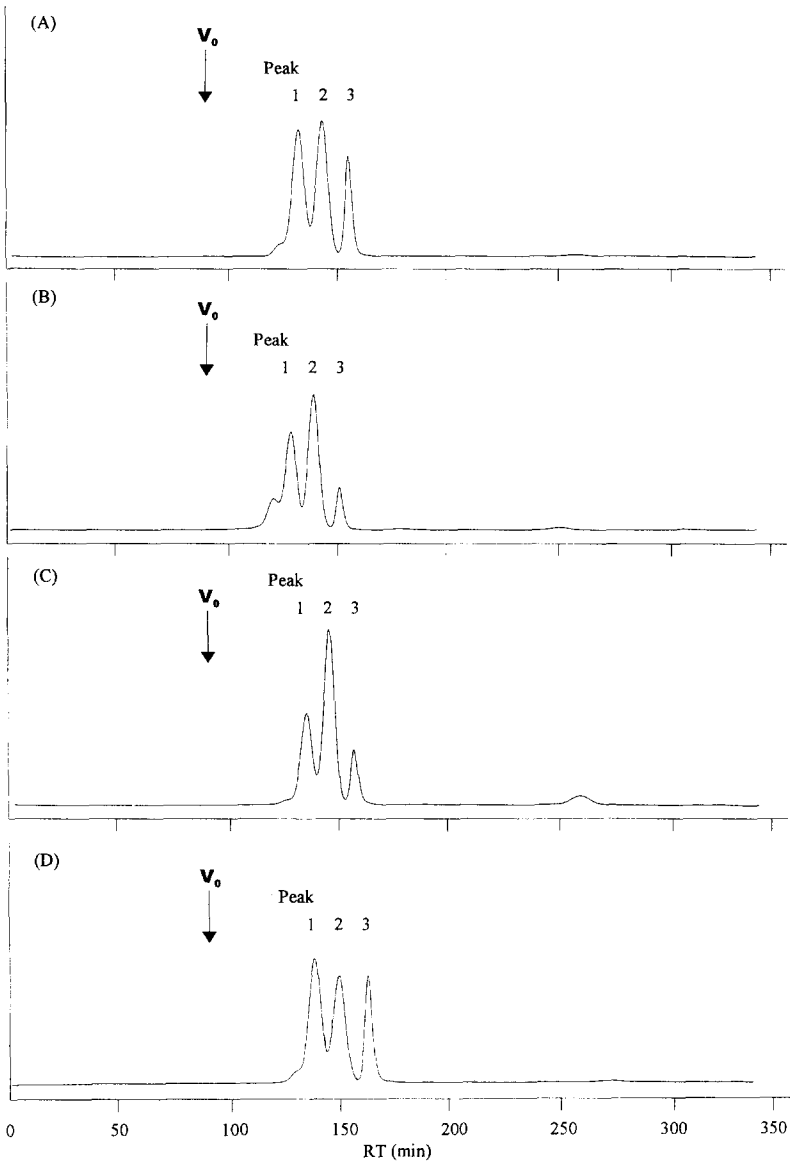

Fig. 4. Elution profiles of the reaction products from Alg-Na (A), polyM (B), polyG (C) and MG random (D) blocks generated by the *Alteromonas* alginate lyase. Substrate solutions containing 6 to 7 mg/mL of each in 0.1 M Tris-HCl buffer (pH 7.5) were incubated for 12 h at 30 °C with the 150 U/mL purified alginate lyase of *Alteromonas* sp. H-4. The reaction mixture was applied to a column (1.34 cm × 40 cm) of Cellulofine GCL25m (Seikagaku Corporation) and eluted with 0.1 M Na_2SO_4 at 25 mL/h. The reaction products from substrate depolymerization were detected at 235 nm using a Hitachi L-4200 UV-VIS detector. The arrow indicates the void volume of the column.

Table 3
Analysis of enzyme products separated by gel filtration chromatography

Peak	Total uronate (μM) [a]				Reducing termini (μM) [b]				DP of oligouronate			
	PolyM	PolyG	MG random	Alg-Na	PolyM	PolyG	MG random	Alg-Na	PolyM	PolyG	MG random	Alg-Na
Peak 1	6.03	4.67	6.11	5.27	0.784	0.641	0.862	0.795	7.7	7.3	7.1	6.6
Peak 2	5.30	6.14	4.85	5.15	1.04	1.07	0.785	0.906	5.0	5.7	6.2	5.7
Peak 3	1.09	0.966	2.02	2.14	0.313	0.293	0.624	0.600	3.5	3.3	3.2	3.6

[a] Total uronate of enzyme products of each substrate was determined using each substrate as standard.
[b] Reducing termini was determined using glucose as standard.

determined to be higher than for the other substrates. The *Alteromonas* enzyme was found to be activated in the presence of 50 mM $MgCl_2$ [14]. In this case, the K_m constants of the enzyme for the polyM block, polyG block, MG random block and Alg-Na were 227 mg/mL, 16 mg/mL, 14 mg/mL and 18 mg/mL, respectively (Table 2). The K_m value for each substrate was considerably lower in the presence of $MgCl_2$. The decrease tended to be similar for the polyM block (15.9%) and polyG block (24.2%), and for Alg-Na (7.9%) and the MG random block (8.5%). The efficiency, estimated from V_{max}/K_m, for each substrate was also improved about 10-fold in the presence of $MgCl_2$, rather than in a Tris-HCl buffer without $MgCl_2$.

The end-products after enzymic degradation of the polyM block, polyG block, MG random block and Alg-Na were separated by Cellulofine GCL 25m gel filtration chromatography, and the elution profiles of unsaturated oligouronates are shown in Fig. 4. Three major peaks (peak 1, 2 and 3) were observed in the enzyme products of each substrate, although a minor peak with a higher DP was detected in the enzyme products of the polyM block (Fig. 4B). Undegraded substrates were eluted at void volume (indicated by an arrow) in the gel filtration column. Elution profiles of enzyme products of the polyM block and polyG block were similar, as were those of the MG random block and Alg-Na. The degree of polymerization (DP) of each peak was estimated by comparison of total uronate residues and reducing termini determined in corresponding fractions to each peaks (Table 3). The estimated DP of peaks 1, 2 and 3 of the polyM block products were 8, 5 and 3, respectively. The estimated DP of the same peaks of the polyG block and the MG random block were 7, 6 and 3. The estimated values of the Alg-Na products of the same peaks were 7, 6 and 4.

The recovery of the various products are summarized in Table 4. About 6 to 7 mg aliquot of uronate substrate was applied to the column. After gel filtration chromatography, the total amount of uronate in all detected peaks having an absorption at 235 nm was determined, except for the peak at void volume. The total amounts of uronate reaction products of the polyM block, polyG block, MG random block and Alg-Na by the bacterial alginate lyase were 7.3 mg, 5.9 mg, 6.9 mg and 7.0 mg, respectively. The estimated recovery was 97.3% for the polyM block, 98.3% for the polyG block, 98.6% for the MG random block and 100% for the Alg-Na. The recovery of reaction products for the abalone alginate lyase was 88.0% for the polyM block, 74.3% for the MG random block and 64.3% for the Alg-Na (Table 4).

3. Discussion

An alginate lyase purified from the culture supernatant of the marine bacterium *Alteromonas* sp. H-4 was shown to be capable of degrading not only the MG random block and Alg-Na but also the polyM block and polyG block (Fig. 2). Moreover, the results from activity staining using substrate-containing gel

Table 4
Recovery of uronates separated by gel filtration chromatography of the alginate lyase products

Substrate	The H-4 alginate lyase				Abalone alginate lyase			
	Alg-Na	PolyM	PolyG	MG random	Alg-Na	PolyM	PolyG	MG random
Applied amounts of uronate (mg)	7.0	7.5	6.0	7.0	7.0	7.5	NT [a]	7.0
Total amounts of uronate after gel filtration (mg)	7.0	7.3	5.9	6.9	4.5	6.6	NT	5.2
Recovery (%)	100	97.3	98.3	98.6	64.3	88.0	NT	74.3

[a] Not tested.

overlays after isoelectric focusing of the alginate lyase indicated that the enzyme was a single protein capable of degrading both the polyM block and polyG block (Fig. 3). Three major unsaturated oligouronides with DP of 7–8, 5–6 and 3–4 were detected close to 100% as end-products of the polyM block, polyG block, MG random block and Alg-Na (Fig. 4 and Table 3). Recovery of oligouronides generated from all substrates by the bacterial enzyme was in every case higher than those of the polyM specific abalone enzyme (Table 4). This suggests that the bacterial alginate lyase seemed to almost completely degrade alginate molecules. We attribute the unlimited substrate specificity of the *Alteromonas* enzyme to the higher protoplast isolation efficiency from *Laminaria* rather than that by the use of abalone enzyme [16].

Substrate specificities of the alginate lyases are defined in terms of a preference for polyM block or polyG block. Alginate lyases from abalone hepatopancreas (used in this study), *Photobacterium* sp. [6], and *Pseudomonas aeruginosa* [7] are reported to be polyM specific lyases, while *Klebsiella aerogenes* alginate lyase is well known to be specific for polyG [8]. There has been only one report on an alginate lyase having preferential degradation for both polyM and polyG blocks [21]. This alginate lyase was produced from *Bacillus circulans*. The activity of *Bacillus* alginate lyase towards the polyM block increased remarkably with the addition of Ca^{2+} [12]. Although the effects of Ca^{2+} on the enzyme activity of the *Alteromonas* sp. H-4 for alginate block structures have not been investigated, the *Alteromonas* enzyme was found to be activated in the presence of Mg^{2+} [14]. A decrease of K_m and an increase of the initial rate of the *Alteromonas* enzyme for each substrate were observed in the presence of 50 mM $MgCl_2$. The decrease of K_m for polyM block and polyG block were almost the same (Table 2). No remarkable differentiated responses for Mg^{2+} to substrate specificity were observed in the *Alteromonas* enzyme, such as is seen in the *Bacillus* enzyme for Ca^{2+}. Furthermore, no homology of *N*-terminal amino acid sequence was observed between the *Alteromonas* enzyme and other known alginate lyases (Table 1). These results suggest that the *Alteromonas* extracellular alginate lyase may be a unique enzyme with new type of substrate binding, showing no preference for degradation of either type of alginate blocks.

The K_m of the alginate lyase of *Alteromonas* sp. H-4 for polyM block is higher than that for other alginate blocks (Table 2). These results indicate that affinities of the enzyme may be different for polyM block and polyG block. As pointed out by Gacesa in his 1992 review [1], the substrate specificity of alginate lyases has been determined only for homopolymeric-enriched fractions, and the degradation ability of the enzyme for heterogenous linkages contained in these homopolymeric fractions has been ignored. Haugen et al. [5] analyzed the sequence specificity recognized by 'polyG specific' enzyme from *Klebsiella* and 'polyM specific' enzyme from *Haliotis* using a combination of kinetic measurements with a well-characterized substrate and end-group analysis of reaction products. They concluded that the *Haliotis* enzyme will cleave M–M, G–M and M–G linkages, while *Klebsiella* enzyme will cleave G–M and G–G linkages. Since the abalone enzyme showed little activity for the polyG block used in this study (Fig. 2), this suggests there are few M–M, M–G and G–M linkages contained in the polyG block.

4. Experimental

Bacterial strain.—An alginolytic marine bacterium, strain H-4, isolated from decaying *Laminaria* fronds was used in this study. The properties of this bacterium have been described in a previous report, in which the bacterium was assigned to the genus *Alteromonas* and shown to utilize sodium alginate as its sole carbon source [12]. The bacterium was maintained at 20 °C in a slant culture using an AI2 agar medium (pH 7.8) containing 0.4% sodium alginate, 0.1% NH_4Cl, 0.01% yeast extract, 1.5% agar and 75% seawater.

Substrate.—Sodium alginate (Alg-Na; 300–400 cP) was purchased from Wako Pure Chemical Industries, Ltd. PolyM block, polyG block and MG random block were prepared from sodium alginate by mild hydrolysis according to Haug et al. with minor modifications [22]. The composition of each block was quantified with a ^{1}H-nuclear magnetic resonance spectrometer (JEOL FX-90Q) according to the methods of Penman and Sanderson [23] and Grasdalen et al. [24]. (Table 5) Each substrate (10 mg) was dissolved in D_2O (0.4 mL) and H^1-NMR spectra were run at 89.55 MHz on a JEOL FX-90Q. The spectra were recorded at 90°C. Numerical value of frequency (Fn) of M/G ration and the doublet frequencies (Fnn) were calculated by the formula of Grasdalen et al. [24].

A purified extracellular alginate lyase.—An extracellular alginate lyase was purified from the culture supernatant of the strain by gel-filtration and anion-exchange chromatography as previously described by

Sawabe et al. [14]. The homogeneity of the enzyme was confirmed by native-, SDS-polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF). Native- and SDS-PAGE were performed using one-dimensional 1.0 mm thick slab gels of 7.0% acrylamide/0.187% bis-acrylamide and 10% acrylamide/0.27% bis-acrylamide, respectively, as separation gels. The native-PAGE sample was loaded in a cold room (4 °C). A commercially available polyacrylamide gel plate (PhastGel p*I* 3–10, Pharmacia Biotech) was used for isoelectric focusing of the enzyme using a PhastSystem™ (Pharmacia Biotech). IEF was performed according to the recommendations of the manufacturer. After electrophoresis, the gels were stained with Coomassie brilliant blue (CBB) R-250 to visualize the proteins.

Enzyme assay.—The above prepared enzymes were added to a substrate solution containing 0.1% substrate and 0.1 M Tris-HCl buffer (pH 7.5). Enzyme activity was monitored by measuring the increase in absorbance at 235 nm using a Hitachi spectrophotometer. One enzyme unit was defined as previously described in Sawabe et al. [12,14]. A 0.5% abalone alginate lyase solution was prepared by dissolving abalone acetone powder (Sigma) in 0.1 M Tris-HCl buffer (pH 7.5) and removing undissolved debris by centrifugation (10,000 × *g*, 5 min). This solution was used as a reference of polyM specific lyase to investigate the substrate specificity.

Detection of enzyme activity on gels.—Enzyme activities in IEF gel were detected using the substrate-containing agar gel overlay technique described by Caswell et al. [25], with some modifications. The overlays were prepared by dissolving 1.5% (w/v) agar (Wako Pure Chem. Industries) and 0.5% sodium alginate (Alg-Na) in 0.1 M Tris-HCl buffer (pH 7.5). The agar overlays were cast using the same apparatus that made the 1.0 mm thick slab gels for PAGE, and solidified at room temperature. For determining the substrate specificity of the enzyme, each of polyM block and polyG block-containing gel overlay was prepared as same manner to make the Alg-Na-containing overlay.

After electrophoresis, the enzyme activities of substrate-containing agar overlays were detected in the same manner described by Caswell et al. [25], except cetylpyridinium chloride solution (Sigma) was used instead of ruthenium red. The substrates-containing overlays and the electrophoresised gel were sandwiched together and placed in a humidor at 30 °C for 1–3 h to allow degradation of these substrates by the enzyme. The overlays were immersed in 10% cetylpyridinium chloride to detect the alginate degrading activities.

N-Terminal amino acid sequence.—Purified extracellular alginate lyase of *Alteromonas* sp. strain H-4 was applied to the SDS-PAGE, and then the protein was electroblotted to an Immobilon™-PSQ^{SQ} PVDF membrane (Millipore) using 10 mM 3-cyclohexylaminopropanesulfonic acid (CAPS) containing 10% MeOH (pH 11.0). Electroblotting occurred at 200 mA for 1 h. The membrane was stained with CBB R-250 to visualize the protein. The protein band was cut and air dried, and stored at −80 °C until analysis. An Applied Biosystems Model 473A gas phase protein sequencer was used for automated Edman degradation.

Determination of K_m.—K_m and V_{max} data were obtained by continuously monitoring the absorbance at 235 nm of the depolymerization reactions at 30 °C in 10 mm quartz cuvettes. Measurements were made using a Hitachi 200-20 model spectrophotometer with a chart recorder (Hitachi 056 Model two-pen recorder). Substrate concentrations ranged from 0.1 mg/mL–2.0 mg/mL, and substrates were dissolved in 0.1 M Tris-HCl buffer (pH 7.5). A Tris-HCl buffer containing 50 mM $MgCl_2$ was also used for the assay. Apparent initial velocities were maintained for sufficient periods of time (at least 15 min) to allow their direct graphical interpretation.

Analysis of products from depolymerization of alginates by the extracellular alginate lyase.—Substrate solutions containing 6 to 7 mg/mL Alg-Na, polyM, polyG or MG random blocks in 0.1 M Tris-HCl buffer (pH 7.5) were incubated for 12 h at 30 °C with either 150 U/mL purified alginate lyase of *Alteromonas* sp. strain H-4 or 130 U/mL abalone alginate lyase (1.0% solution in 0.1 M Tris-HCl), respectively. No further increase in absorbance at 235 nm could be detected after this time. The solution was applied to a column (1.34 cm × 40 cm) of Cellulofine GCL25m (Seikagaku corporation) and the products were eluted with 0.1 M Na_2SO_4 at 25 mL/h according to Haugen et al. [5]. The reaction products were detected at 235 nm using a Hitachi L-4200 UV-VIS detector and simultaneously collected into 1.2 mL aliquots. Total uronate of each fraction was quantified by phenol-sulfate determination [26] using each substrate as a standard. The reducing power of each peak with ultraviolet absorption was determined by the Somogyi–Nelson method [27] using glucose as the standard. The DPn of each peak was estimated by comparing the total amount of uronate and the reducing termini.

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