

# A new high-alkaline alginate lyase from a deep-sea bacterium *Agarivorans* sp.

Tohru Kobayashi · Kohsuke Uchimura ·  
Masayuki Miyazaki · Yuichi Nogi · Koki Horikoshi

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**Abstract** A high-alkaline, salt-activated alginate lyase is produced by *Agarivorans* sp. JAM-A1m from a deep-sea sediment off Cape Nomamisaki on Kyushu Island, Japan. Purified to homogeneity, as judged by SDS-PAGE, the enzyme (A1m) had a molecular mass of approximately 31 kDa. The optimal pH was around 10 in glycine–NaOH buffer, and the activity was increased to 1.8 times by adding 0.2 M NaCl. However, when the optimal pH in the presence of 0.2 M NaCl was shifted to pH 9.0, the activity was more than 10 times compared with that at pH 9 in the absence of NaCl. A1m showed the optimal temperature at around 30°C and was stable to incubation between pH 6 and 9. The enzyme degraded favorably mannuronate–guluronate and guluronate-rich fragments in alginate. Shotgun cloning and sequencing of the gene for A1m revealed a 930-bp open reading frame, which encoded a mature enzyme of 289 amino acids (32,295 Da) belonging to polysaccharide lyase family 7. The deduced amino acid sequence showed the highest similarity to that of a *Klebsiella* enzyme, with only 54% identity.

**Keywords** Alginate lyase · PL family 7 · Alkaline enzyme · Deep sea · *Agarivorans*

## Introduction

Alginate is a linear hetero-polyuronic acid consisting of 1,4 linked  $\alpha$ -L-guluronic acid (G) and its C5 epimer,  $\beta$ -D-mannuronic acid (M). These residues are arranged in block structures that comprise a homopolymeric G block, M block, alternating MG (GM) block, and heteropolymeric MG (GM) blocks (Gacesa 1988). Alginates are synthesized as a cell wall component by brown seaweeds and also as an exopolysaccharide by bacteria such *Pseudomonas aeruginosa* (Evans and Linker 1973) and *Azotobacter vinelandii* (Gorin and Spencer 1966).

Unlike pectate or pectin decomposing enzymes, alginate-degrading enzymes are known to be only lyases (transeliminase) even if they are intracellular oligo-alginate-degrading enzymes. Alginate lyases have been isolated from various sources, namely, marine algae, marine mollusks, fungi, bacteria, bacteriophages, and viruses (Wong et al. 2000). They are classified into three groups by their substrate specificity toward G block (polyguluronate lyase; EC4.2.2.11), M block (polymannuronate lyase; EC4.2.2.3), or MG block (Wong et al. 2000). Furthermore, they are also grouped into three types based on their molecular masses: small (25–30 kDa), medium-sized (around 40 kDa), and large lyases (>60 kDa) (Osawa et al. 2005). Thus, it is easily supposed that alginate lyases show a wide molecular diversity. Indeed, alginate lyases are classified into seven polysaccharide lyase (PL) families ([http://www.cazy.org/fam/acc\\_PL.html](http://www.cazy.org/fam/acc_PL.html)). Many alginate lyases have optimal pH values between pH 7 and 8, in contrast to the optimal high-alkaline pH values of pectate lyases (Wong et al. 2000). Alkaline alginate lyases are very few, and the most alkaline enzyme reported to date is derived from the *Chlorella* virus, CVN1 (Suda et al. 1999) with an optimal pH of 10.5; however, there is no report of

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T. Kobayashi (✉) · K. Uchimura · M. Miyazaki · Y. Nogi · K. Horikoshi  
Extremobiosphere Research Center,  
Japan Agency for Marine-Earth Science and Technology  
(JAMSTEC), 2-15 Natsushima, Yokosuka 237-0061, Japan  
e-mail: kobayashit@jamstec.go.jp; tkob@beige.plala.or.jp

its substrate specificity. Other lyases with highly alkaline optimal pH of 9.2–9.6 that degrade the M block as a substrate have been isolated from the invertebrates, *Aplysia depilans* (Boyen et al. 1990b) and *Turbo cornutus* (Muramatsu et al. 1984). Among alginate lyase-producing bacteria, only one alkaliphilic *Bacillus* sp. No. M-2 has been reported to produce an alkaline enzyme, with an optimal pH of 9 (Horikoshi and Akiba 1982). The enzyme is salt-activated and degrades the M block in alginate. This means that isolation of a highly alkaline G or MG block-degrading enzyme from a microorganism has never been reported. Recently, we found a new highly alkaline alginate lyase (A1m) from a deep-sea bacterium, *Agarivorans* sp. JAM-A1m. In this article, we describe screening and purification of the enzyme and cloning and sequencing of the gene for the enzyme.

## Materials and methods

### Isolation of alginate lyase-producing bacteria

Deep-sea sediments were collected off Cape Nomamisaki, at the southwestern tip of Kyushu Island (representative sampling site: 31°18.522N, 129°59.372E), Japan, at a depth of 254 m in July 2005. Aliquots of the samples were spread onto marine agar 2216 (Difco) containing 0.2% sodium alginate (from *Laminaria*, 80–120 cps; Wako Pure Chemicals). After cells had grown at 30°C for 24 h, a 0.8% agar solution containing 0.2% sodium alginate and 50 mM MOPS buffer (pH 7) was poured over the agar. After incubation at 30°C for 24 h, 0.1% cetylpyridinium chloride solution was flowed onto the soft agar. Colonies that formed clear zones were picked up and purified several times on marine agar 2216. The isolates were propagated aerobically in large test tubes containing 5 ml of marine broth 2216 (Difco) plus 0.2% sodium alginate at 30°C for 24 h. Alginate lyase activities in the supernatants were measured in 100 mM acetate buffer (pH 5), MOPS buffer (pH 7), or glycine–NaOH buffer (pH 9) at 30°C for 1 h. The alginate lyases of four isolates among ten candidates showed higher activity in glycine–NaOH buffer (pH 9). One of the isolates, strain JAM-A1m, showed the highest productivity, and the enzyme showed the highest activity at pH 10 in glycine–NaOH buffer.

### Determination of 16S rRNA gene sequence of isolate

To clarify the phylogenetic position of strain A1m, the 16S rRNA gene sequence was determined by colony PCR. The gene was amplified using bacterial primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGCTACCTTGTTACGACTT-3'), and a small pieces

of strain A1m as the template in a DNA thermal cycler (Gene Amp PCR system 9700, ABI PRISM) with an LA Taq DNA polymerase. PCR conditions were 2 min at 96°C, followed by 30 cycles of 30 s at 96°C, 30 s at 55°C, and 90 s at 72°C, and final extension for 7 min at 72°C. After purification of the amplified gene fragment, sequencing was done with a DNA sequencer (Mega BACE 1000, GE Healthcare) using a DYEnamic ET Terminal Sequencing kit (GE Healthcare). The nucleotide sequence of the 16S rRNA gene has been submitted to GenBank/EMBL/DDBJ databases under accession no AB426125. Strain A1m has been deposited at the National Institute of Advanced Industrial Science and Technology (AIST) under deposit number FERM P-21397.

### Production and purification of A1m

*Agarivorans* sp. JAM-A1m was propagated aerobically, with shaking, in 100 ml of medium optimized for production of A1m at 30°C for 24 h. The medium was composed of (w/v) 0.5% polypeptone S (Nihon Pharmaceutical), 0.5% yeast extract (Difco), 2% NaCl, 0.2% sodium alginate, 0.02% MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01% K<sub>2</sub>HPO<sub>4</sub>, and 0.002% MnCl<sub>2</sub>. Sodium alginate was indispensable for the production of A1m, because the enzyme was inducible. The spent media were centrifuged (8,000×g for 20 min at 4°C), and the supernatant (2.5 l) was concentrated to 290 ml using a hollow fiber filter (AIP0013, 6 kDa cut off, Asahikasei). The concentrate was applied to a SuperQ Toyopearl column (2.5 × 8 cm; Tosoh) equilibrated with 50 mM Tris–HCl buffer (pH 8.0). The absorbed proteins were eluted with a 450-ml linear gradient of 0–0.22 M NaCl in the equilibrating buffer. Alginate lyase activity was eluted around 0.16 M NaCl. Ammonium sulfate was added to the pooled fraction (75 ml) to a final concentration of 2 M. The solution was applied to a Butyl Toyopearl column (1.5 × 5 cm; Tosoh) equilibrated with 10 mM Tris–HCl buffer (pH 8.0) containing 2 M ammonium sulfate. Proteins were eluted with a 120-ml linear gradient of 2–0.6 M ammonium sulfate in 10 mM Tris–HCl buffer. The active fractions were obtained at around 1.4 M ammonium sulfate. The active fractions were combined, and the same procedures were repeated except that a linear gradient of 2–0.5 M ammonium sulfate was used. The active fractions were concentrated and desalted by ultrafiltration using a YM3 membrane (Millipore).

### Determination of molecular mass of A1m

Molecular mass of the purified enzyme was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and gel permeation chromatography. SDS-PAGE was performed by the method of Laemmli (1970) using a 12.5%

acrylamide slab gel (Bio-Rad). The proteins were stained with Coomassie Brilliant Blue R-250. Precision plus protein standards (Bio-Rad) were used as the molecular mass markers. Bio-Gel-A0.5 m gel column (1.5 × 75 cm; Bio-Rad) was equilibrated with 0.2 M KCl in 10 mM Tris–HCl buffer (pH 7.5). The standard proteins used were conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.2 kDa).

#### Analysis of N-terminal amino acid sequence

After SDS-PAGE, the protein band was electrotransferred onto a polyvinylidene difluoride membrane (Imobulon; Millipore) that had been wetted with methanol. The amino acid sequences were determined by a protein sequencer (model 497HT; Applied Biosystems).

#### Enzyme assay and protein content

Alginate lyase activity was measured in a 0.5 ml of reaction mixture composed of 0.2% (w/v) sodium alginate, 100 mM glycine–NaOH buffer (pH 10), and a suitably diluted enzyme solution. After incubation at 30°C for 15 min, the reaction was terminated by adding 2 ml of 5 mM HCl, and then the absorbance of the solution was measured at 235 nm. One unit of enzyme activity was defined as the amount of protein that increased absorbance by 0.1 per min at 235 nm. To confirm the products of the enzymatic reaction, thiobarbituric acid (TBA) test was used according to Weissbach and Hurwitz (1959). The viscometric assay was done using as Ostwald viscometer (No.1; Shibata Scientific Technology). The reaction mixture included 0.2% sodium alginate, 100 mM glycine–NaOH buffer (pH 9), 200 mM NaCl, and a suitably diluted enzyme solution in a total volume of 10 ml. The decrease of viscosity in the mixture was measured at 30°C at timed intervals. The absorbance at 235 nm ( $A_{235}$ ) of timed sample (0.5 ml) was also measured after adding 2 ml of 5 mM HCl. The protein content was quantified by a DC protein assay kit (Bio-Rad) using bovine serum albumin as the standard.

#### Preparation of substrates

Mannuronic acid (M) and guluronic acid (G) blocks were prepared by oxalic acid hydrolysis of sodium alginate basically according to the method of Haug et al. (1966). M and G blocks were qualitatively confirmed by infrared spectroscopy (type JIR-7000; JEDR). For the G block, the absorptions around 1,415, 1,324, 1,124, 1,091, 1,027, and 949  $\text{cm}^{-1}$  showed greater intensity, whereas the intensities of the M block around 1,144, 1,039, and 820  $\text{cm}^{-1}$  were clearer and larger than those for G block (Sartori et al. 1997;

Pereira et al. 2003). MG blocks were prepared from partial acid hydrolysis of 5% (w/v) sodium alginate in 0.3 N HCl at 100°C for 20 min as described by Haug et al. (1967).

#### Cloning and sequencing gene for A1m

The genomic DNA of *Agarivorans* sp. JAM-A1m, which was prepared by the method of Saito and Miura (1963), was digested with *Sau3AI*. DNA fragments (0.75–3 kb) were ligated into *Bam*HI site of pUC18. *Escherichia coli* DH5 $\alpha$  was transformed by the resultant plasmids. After transformants were grown on Luria-Bertani agar containing 0.1% sodium alginate plus 100  $\mu\text{g ml}^{-1}$  ampicillin, a soft agar solution (0.2% alginate, 0.2 M NaCl, 50 mM glycine–NaOH buffer, and 0.8% agar, pH 9) was poured over the agar plate. The agar was incubated at 30°C for 24 h, and then 0.1% cetylpyridinium chloride solution was poured onto the soft agar. Transformants showing alginate-degrading zones were picked up and purified several times on LB agar containing 100  $\mu\text{g ml}^{-1}$  ampicillin. Several plasmids were isolated from the cultured transformants by a High Pure Plasmid Isolation kit (Roche), and subsequently inserted DNA fragments were amplified by PCR using primers A, 5'-CAAGGCGATTAAGTTGGGTA ACG-3' and B, 5'-CTTCCGGCTCGTATGTTGTGTG-3', which were designed from the outside of multiple cloning sites in the pUC18 plasmid. The amplified fragments were ligated into a pCR2.1 using a TA Cloning kit (Invitrogen) and were transformed into *E. coli* TOP10. After blue/white colony selection of transformants, cloned DNA fragments were amplified by colony PCR using primers A and B and were sequenced using a DYEnamic ET Terminal Sequencing kit and a DNA sequencer. The A1m gene was also sequenced and confirmed by PCR using primers that were designed from the above sequence and the genomic DNA of *Agarivorans* sp. JAM-A1m as the template. Nucleotide sequence data of A1m gene have been submitted to the GenBank, EMBL, and DDBJ databases under accession no AB426616.

## Results

#### Analysis of 16S rRNA gene sequence of strain JAM-A1m

A 1,497-bp fragment of the 16S rRNA gene of strain JAM-A1m was sequenced and compared with those of other strains. The highest nucleotide sequence match was that of *Agarivorans albus* MKT87 (AB076559) with 98.6% identity (1,453 of 1,473 bases match). The next best match was observed on that of *Agarivorans* sp. JAMB-A11 (AB167508) with 98.4% identity (1,411 of 1,434 bases

**Table 1** Summary of purification of A1m

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units mg <sup>-1</sup> )	Fold	Yield (%)
Broth	2,500	4,925	700	0.142	1	100
Concentrate	290	217.5	345.1	1.59	11.2	49.3
SuperQ Toyopearl	75	27	254.3	9.42	66.3	36.3
First Butyl Toyopearl	25	0.7	45.5	65.0	457.8	6.5
Second Butyl Toyopearl	11.5	0.4	43.4	108.5	764.1	6.2

match). These results indicate that strain JAM-A1m is closely related to the genus *Agarivorans*.

#### Purification and physicochemical properties of A1m

A1m was purified to homogeneity as judged by SDS-PAGE from spent media of *Agarivorans* sp. JAM-A1m. A typical purification was summarized in Table 1. The purification increased the specific activity 764 times with 6% recovery of the initial activity. The molecular mass of the enzyme was around 31 kDa by SDS-PAGE (Fig. 1). A1m was eluted at the same position of carbonic anhydrase by Bio-Gel-A0.5m gel filtration column chromatography, indicating that the enzyme is monomer. The N-terminal amino acid sequence of the purified A1m was Ala-Thr-Thr-Thr-Pro-Ala-Glu-Val-Leu-Asp-Leu-Ser-X-X-X-Val-Thr, where X is an unidentified amino acid residue.

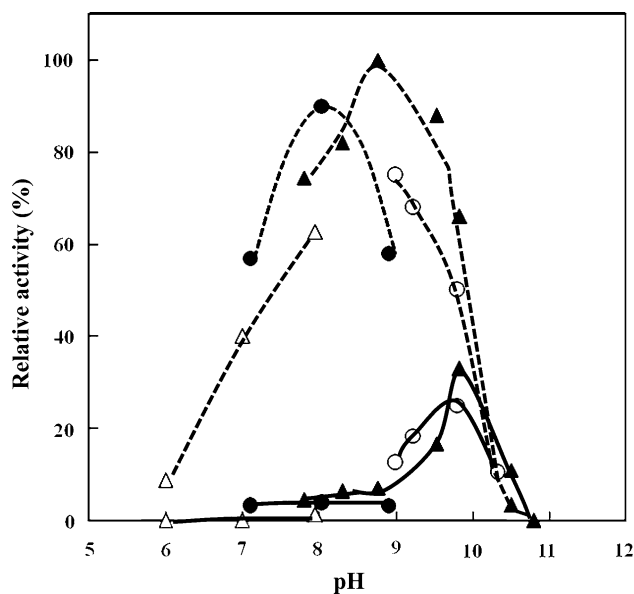
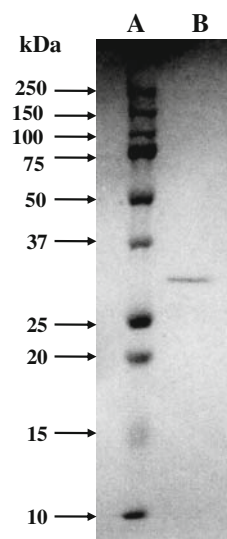
#### Effects of pH and NaCl on A1m

The optimal pH of A1m activity was around pH 10 in 100 mM glycine–NaOH buffer. Very low activity was observed between pH 6 and 11 except around pH 10. When 0.2 M NaCl was added to the reaction mixture, the activity was increased to 1.8 times the original activity, and then the optimal pH was shifted to pH 9 in glycine–NaOH buffer. The activity was increased to more than 10 times that at pH 9 in the absence of NaCl. Similar results were observed in 100 mM borate buffer with or without 0.2 M NaCl, as shown in Fig. 2. In contrast, the stability of A1m in various pH buffers was unaffected by adding NaCl. A1m was quite stable in incubation at 30°C for 1 h between pH 6 and 9 (Fig. 3). Although the residual activities were only 60% of those in the absence of NaCl, the enzyme was modestly stable between pH 6 and 7 under the same conditions in the presence of 0.2 M NaCl.

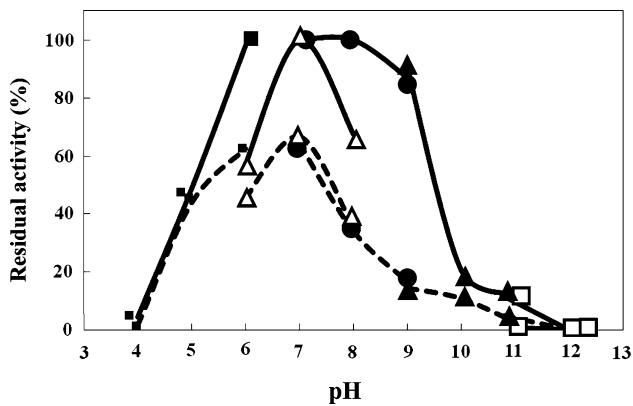
#### Effects of NaCl on A1m

The effect of the NaCl concentration on the enzyme activity was examined. A1m activity at pH 9 in glycine–NaOH

**Fig. 1** SDS-PAGE of purified A1m. Lane A protein mass markers; lane B purified enzyme (5 µg). The proteins were subjected to electrophoresis on 12.5% acrylamide gel and stained with Coomassie Brilliant Blue R-250



**Fig. 2** Effect of pH on A1m activity. The activity was measured at 30°C for 15 min in a total volume of 0.5 ml containing 2.2 or 1.1 µg of purified A1m in the absence (solid line) or presence of 0.2 M NaCl (dotted line), respectively. The following 100 mM buffers were used: open triangles MOPS (pH 6–7.9); filled circles Tris–HCl (pH 7.1–8.9); open circles borate–NaOH (pH 9–10.3); filled triangles glycine–NaOH (pH 8–10.8). The highest activity, obtained at pH 9 in glycine–NaOH buffer plus 0.2 M NaCl, is taken as 100%

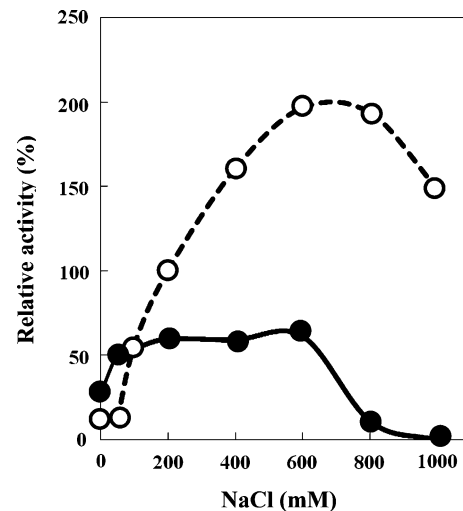


**Fig. 3** Effect of pH on A1m stability. Purified A1m (1  $\mu$ g) was preincubated at 30°C for 1 h in various 20 mM buffers with (dotted line) or without (solid line) 0.2 M NaCl. After preincubation, the residual activity was measured at 30°C for 15 min in 100 mM glycine–NaOH buffer (pH 9) plus 0.2 M NaCl. The buffers used were acetate (pH 4–6, filled squares), MOPS (pH 6–8, open triangles), Tris–HCl (pH 7–9, filled circles), glycine–NaOH (pH 9–11, filled triangles), and phosphate–NaOH (pH 11–12.3, open squares). The highest residual activity treated in 20 mM Tris–HCl (pH 7) is taken as 100%

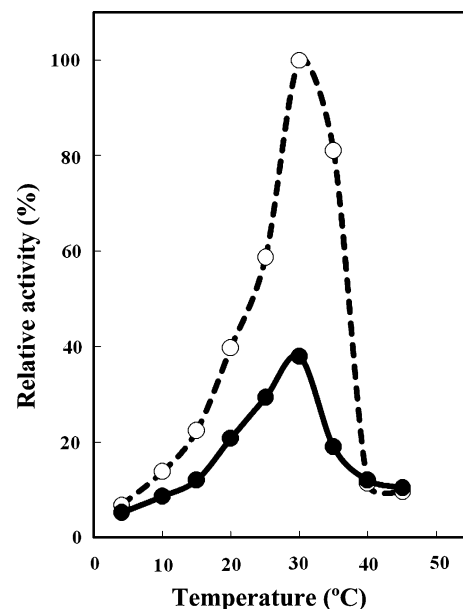
buffer increased with increasing NaCl concentration, and the activity reached maximum at 0.6–0.8 M NaCl, which was around 20 times without NaCl (Fig. 4). Although the effect of NaCl on activation of A1m activity at pH 10 was less than that at pH 9, the activity increased within the broad concentration of 0.1–0.6 M NaCl to approximately double that without NaCl (Fig. 4). Thus, A1m is one of the most salt-activated enzymes among alginate lyases reported to date (Horikoshi and Akiba 1982; Kitamikado et al. 1992; Wong et al. 2000; Xiao et al. 2006). The kinetic parameters for alginate degradation in the presence or absence of NaCl at pH 9 were determined by double reciprocal Lineweaver Burk plots. The apparent  $K_m$  values were 0.032% regardless of whether NaCl was present or not. The apparent  $V_{max}$  values were 38.4, 285.7, 416.7, and 526.3 units  $\text{mg}^{-1}$  protein in the presence of 0, 0.1, 0.2, and 0.5 M NaCl, respectively. These results suggest that the catalytic efficiency of A1m for alginate degradation is increased by adding NaCl.

#### Effects of temperature of A1m

The optimal temperature for the reaction of A1m was around 30°C either in the presence of 0.2 M NaCl at pH 9 or in its absence at pH 10. Activation of the enzyme by NaCl was prominent between 25 and 35°C (Fig. 5). As expected, A1m is quite thermolabile. A1m is rapidly inactivated upon incubation at 30–50°C for 15 min in 20 mM glycine–NaOH buffer (pH 9) with or without 0.2 M NaCl. The residual activity after treatment at 30°C with 0.2 M NaCl was only 53% of the residual activity



**Fig. 4** Effect of NaCl concentration on A1m activity. The activity was measured at 30°C for 15 min in 100 mM glycine–NaOH buffer with the indicated NaCl concentrations. The purified enzyme (2.7 or 1.1  $\mu$ g) was used in a reaction mixture at pH 10 (filled circles with solid line) or at pH 9 (open circles with dotted line). The activity at pH 9 in glycine–NaOH buffer plus 0.2 M NaCl is taken as 100%



**Fig. 5** Effect of temperature on A1m activity. Alginate lyase activity was measured in 100 mM glycine–NaOH buffer at the indicated temperatures for 15 min with 2.2 or 1.1  $\mu$ g of purified enzyme in the absence (pH 10, filled circles with solid line) or presence of 0.2 M NaCl (pH 9, open circles with dotted line), respectively. The maximal activity in the presence of 0.2 M NaCl is taken as 100%

without NaCl. However, relative inactivation rates were similar whether NaCl was present or not. The residual activities after treatment at 50°C were only 10% of the initial activities after incubation at 30°C with or without NaCl.

### Effects of other salts and metal ions on A1m activity

A1m was activated by several salts, including 0.2 M each NaF, NaBr, NH<sub>4</sub>Cl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, KCl, and K<sub>2</sub>HPO<sub>4</sub> (Table 2). Pd<sup>2+</sup>, Hg<sup>2+</sup>, and Sr<sup>2+</sup> (1 mM each) ions inhibited the enzyme by 95, 80, and 46%, respectively. Ca<sup>2+</sup> and Mg<sup>2+</sup> ions did not affect enzyme activity unlike other alginate lyases (Wong et al. 2000; Svanem et al. 2001; Xiao et al. 2006). NaCl was the best activator among tested salts and metal ions. The enzyme was strongly inhibited by 5 mM EDTA and EGTA (Table 2). Dithiothreitol and 2-mercaptoethanol (5 mM each) did not affect the enzyme activity at all.

### Substrate specificity

A1m preferably degraded the MG and G blocks to the M block. The relative activities for alginate, MG, G, and M blocks were 100, 131.7 ± 16.5, 83.3 ± 16.9, and 27.3 ± 13.4%, respectively, under the standard assay conditions at pH 10. The mean value and standard deviation were calculated from four experimental results. Similar results were observed when measured at pH 9 with 0.2 M NaCl.

**Table 2** Effects of salts and metal ions on A1m activity

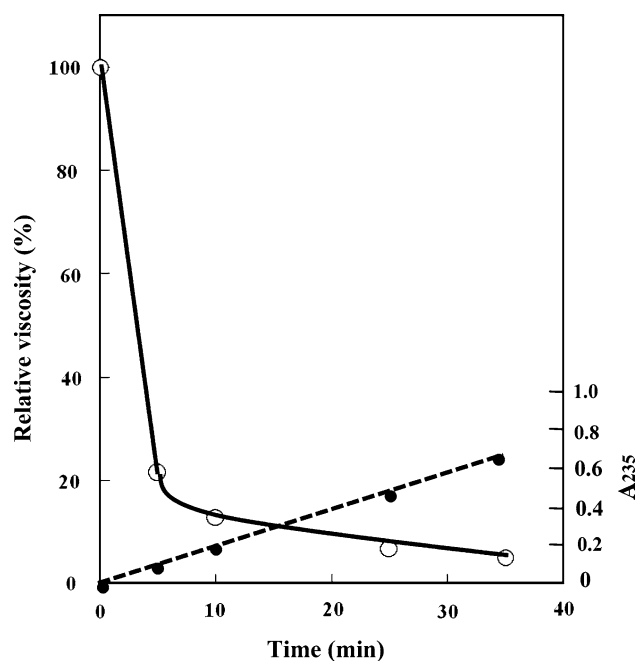
Salts and metals	Conc (mM)	Relative activity (%) <sup>*</sup>
None	–	100
NaCl	200	823
NaBr	200	712
NH <sub>4</sub> Cl	200	549
KCl	200	521
K <sub>2</sub> HPO <sub>4</sub>	200	439
NaF	200	419
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	200	409
CH <sub>3</sub> COONH <sub>4</sub>	200	298
CH <sub>3</sub> COONa	200	233
CaCl <sub>2</sub>	1	76
MnCl <sub>2</sub>	1	72
MgCl <sub>2</sub>	1	101
CoCl <sub>2</sub>	1	102
PdCl <sub>2</sub>	1	5
SrCl <sub>2</sub>	1	54
HgCl <sub>2</sub>	1	29
NiCl <sub>2</sub>	1	106
ZnCl <sub>2</sub>	1	111
CuSO <sub>4</sub>	1	93
EDTA	5	14
EGTA	5	14

<sup>\*</sup> The activity without salts or metals is taken as 100%

The viscosity of an alginate solution rapidly decreased by adding A1m, whereas the values of A<sub>235</sub> increased very slowly (Fig. 6), suggesting that the enzyme degrades alginate by endo fashion. The products of the enzymatic reaction were positive for TBA method. This means that 4-deoxy-L-erythro-hex-4-enopyranosyluronic acids as the non-reducing terminal moiety are contained in the products. In our assay, it showed good correlation between the values of A<sub>235</sub> per mg protein and those of TBA test (A<sub>550</sub> per mg protein) with a ratio of 0.25 (A<sub>235</sub>/A<sub>550</sub>).

### Nucleotide and deduced amino acid sequences for A1m

The shotgun-cloned DNA fragments contained one open reading frame of 930 bp. It started from an ATG codon and terminated at a TAG codon. The G+C content of ORF was 42.6%. A putative ribosome binding site, with the sequence 5'-TATAGG-3', was found 8 bp upstream of the initiation codon. An inverted repeat sequence was found 20 bp downstream of the termination codon. The free energy value of the stem-loop structure was calculated to be -69.1 kJ mol<sup>-1</sup>. The ORF encoded 309 amino acids



**Fig. 6** Viscosity reduction during enzymatic degradation of alginate. A1m (1.5 µg) was added to the reaction mixture composed of 100 mM glycine-NaOH buffer (pH 9), 0.2% sodium alginate and 0.2 M NaCl in a final volume of 10 ml and, at intervals, viscosity in the solution was measured at 30°C. The initial viscosity of the reaction mixture without enzyme was taken as 100%. At timed intervals, samples (0.5 ml) were also withdrawn from the paranemic reaction mixture, and 2 ml of 5 mM HCl was added, followed by measurement of absorbance at 235 nm. Open circles with solid line rate of viscosity reduction; filled circles with dotted line absorbance at 235 nm

including a putative signal sequence of 20 amino acids, as shown in Fig. 7. In the signal sequence of Met<sup>1</sup> to Ala<sup>20</sup>, several basic amino acid residues after Met<sup>1</sup>, and a helix-broken Gly<sup>12</sup> were found, and Leu<sup>18</sup>-Ser<sup>19</sup>-Ala<sup>20</sup> was supposed to be a signal peptidase recognition sequence. Indeed, the N-terminal amino acid sequence of the purified A1m from *Agarivorans* sp. JAM-A1m was found to be Ala<sup>21</sup> to Thr<sup>37</sup> in the deduced amino acid sequence. Thus, the mature enzyme consists of Ala<sup>21</sup> to Lys<sup>309</sup>, with a calculated molecular mass of 32,295 Da, a value that is close to the 31 kDa of the purified A1m. When suitably aligned, the deduced amino acid sequence of A1m exhibited some homology to those of known alginate lyases belonging to polysaccharide lyase family 7. A1m showed the highest similarity to AlyA (Q059478) of *Klebsiella pneumonia* subsp. *aerogenes* with 53.7% identity. It showed moderate similarity to putative alginate lyases of *Vibrio splendidus* 12B01 (A3UR41), *Saccharophagus degradans* strain 2–40 (Q21HU1), *Plesiocystis pacifica* SIR-1 (A6G2Q0), and *Polaribacter dokdonensis* MED152 (A2TZT7) with 50.7, 49.7, 49.1, and 48.1% identities, respectively, which are merely annotated by genome analysis. These results suggest that A1m is a new alginate lyase.

**Discussion**

There have been many reports of alginate lyases from various origins, such as algae, marine animals, marine bacteria, marine fungi, viruses, and other gram-positive and gram-negative bacteria (Wong et al. 2000; Suzuki et al.

2006; Kawamoto et al. 2006; Iwamoto et al. 2001). In marine bacteria, many alginate lyases have been isolated from members of *Pseudomonas* and *Vibrio* (Wong et al. 2000). This study is the first report that the genus *Agarivorans* from a deep-sea sediment produced an alginate lyase. The *Agarivorans* enzyme (A1m) favorably degraded the MG- and G-blocks in alginate. An alginate lyase from *Pseudomonas* sp. (Min et al. 1977), and AlyVG1, AlyVG2 and AlyVG3 from *Vibrio halioticoli* (Sugimura et al. 2000) also show the similar degradation specificity. The optimal pH of A1m was around pH 10 in both glycine and borate buffers, and it shifted to pH 9 when NaCl was added to the reaction mixture. This is the highest optimal pH reported for alginate lyases from microorganisms. In particular, the value is the highest among MG or G block-degrading enzymes. Interestingly, A1m was remarkably activated by salts, probably the cationic ions, Na<sup>+</sup>, NH<sub>4</sub><sup>+</sup>, and K<sup>+</sup> ions. Although salt-activated G block-degrading lyases, such as the alginate lyases AL-1 and AL-2 of marine bacterium ALG-G (Brown and Preston 1991), and the alginate lyases of *Klebsiella aerogenes* type 25 (Lange et al. 1989), *Pseudomonas alginovora* strain Xo17 (Boyen et al. 1990a), and *Vibrio harveyi* AL-128 (Kitamikado et al. 1992), are known, none of them shifted their optimal pH value when activated by adding salts. Furthermore, A1m was not activated by Ca<sup>2+</sup> and/or Mg<sup>2+</sup> ions unlike the G block-specific degrading alginate lyases from *P. alginovora* strain Xo17 (Boyen et al. 1990a) and *Corynebacterium* sp. ALY-1 (Matsubara et al. 1998). The optimal temperature of A1m was around 30°C, a relatively low optimal temperature among known alginate lyases (Wong et al. 2000). A1m was quite stable in incubation at 30°C for 1 h between pH 6 and

**Fig. 7** Nucleotide and deduced amino acid sequences of A1m. Deduced amino acid residues are represented by *single letters* under the nucleotide sequence. The N-terminal amino acid sequence determined from the purified enzyme is *underlined*. The amino acid sequences that are highly conserved among G-block-degrading enzymes in PL family 7 are *boxed*, and identical amino acid residues are shown in *bold letters*



9, whereas the enzyme was relatively unstable under the same conditions in the presence of 0.2 M NaCl. The reason why the enzyme was unstable in the presence of NaCl is not clear.

Alginate lyases are organized into polysaccharide lyase (PL) families 5, 6, 7, 14, 15, 17, and 18, which show wide diversity compared with other polysaccharide lyases. Recently, the crystal structures of several alginate lyases belonging to PL family 7 have been resolved and the active site and hydrogen network clarified (Osawa et al. 2005; Yamasaki et al. 2004, 2005). Among them, the structures of PL family 7 alginate lyases, namely the G block-degrading ALY-1 of *Corynebacterium* sp. (Osawa et al. 2005) and A1-II' of *Sphingomonas* sp. A1 (Yamasaki et al. 2005), and the M block-degrading PA1167 of *Pseudomonas aeruginosa* (Yamasaki et al. 2004), show the jellyroll  $\beta$ -sandwich fold with a large active cleft. According to their structures and sequences analyses, amino acid residues in active sites of PL family 7 enzymes are well conserved depending on their substrate specificity (see Fig. 7).

A1m is a highly alkaline enzyme compared with the other PL family 7 enzymes. We are interested in alkaline adaptation of proteins. We have proposed three alkaline adaptation-strategies of enzymes. The first strategy aims at shifting the pKa of the catalytic site residues toward alkaline pH. The second one aims at acquiring the higher ratio of surface-exposed Asp + Glu to Lys + Arg for the alkaline enzymes. The last one aims at decreasing Lys and Asp residues and at increasing Arg, His, and Glu residues in the alkaline-adaptation process (Shirai et al. 2008). The Asp + Glu/Lys + Arg (40/33) of A1m is larger than that (33/34) of AlyA of *K. aerogenes* which shows the highest similarity to A1m but is a neutrophilic enzyme (Wong et al. 2000). Although the crystal structures of A1m and AlyA are not resolved, this result is in accordance with the second strategy of alkaline adaptation. A determination of the crystal structure of A1m will be helpful to further clarify the alkaline adaptation mechanism.

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