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Intracellular Alginate-oligosaccharide Degrading Enzyme Activity that is Incapable of Degrading Intact Sodium Alginate from a Marine Bacterium *Alteromonas* sp.

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Intracellular homo- and hetero-polymeric blocks degrading enzyme activity incapable of degrading intact sodium alginate was detected in *Alteromonas* sp. strain H-4. The enzyme activity for polyM and MG blocks was highest during the late log phase of the bacterium and was not induced by the addition of sodium alginate to the culture medium. The activity for MG random block was as high as that for polyM, but that for polyG block was half and that for sodium alginate was one fifth. At least 4 kinds of enzyme activities, a polyM specific, a MG-polyM specific, and two kinds of polyG specific enzymes, were detected from the crude intracellular fraction, but a trace spot for sodium alginate. Analysis of reaction products using a partially purified preparation of the enzyme indicated that the enzyme generated a saturated diuronate and an unsaturated polyuronide from polyM block. These results suggest that the intracellular enzymes can degrade only oligosaccharides generated from high molecular alginate by the extracellular alginate lyase and may have an important role in the alginate metabolism of the bacterium.

Key words: *Alteromonas*, marine bacterium, intracellular alginate-oligosaccharide degrading enzymes, saturated diuronate

Alginate lyases have been isolated from a wide variety of sources, including marine molluscs, bacteria, fungi and marine brown algae. All alginate lyases described to date are capable of degrading intact high molecular alginate. The substrate specificities of these lyases have been examined by determining whether the enzymes show a preference for (1-4)-linked β-D-mannuronate (M) or α-L-guluronate (G) of the alginate polymer.[1,2] There have been no reports of an enzyme showing a preference only for oligosaccharides.

We previously reported on the substrate specificity of the extracellular alginate lyase from a marine bacterium, *Alteromonas* sp. strain H-4.[3] Analysis of the substrate specificity of enzyme for polyM, polyG, MG random blocks and intact sodium alginate indicated that the extracellular alginate lyase is capable of effectively degrading both polyM and polyG blocks, and generates unsaturated tri- through octa-uronate from all of homo- and hetero-polymeric substrates.[3] Although the marine bacterium can utilize alginate as its sole carbon source, the extracellular alginate lyase did not generate monouronate units. Therefore we thought that further degradation of these unsaturated oligouronates may be needed for the effective utilization of alginate by the bacterium. We have tried to screen intracellular alginate degrading activities of the bacterium using intact sodium alginate as a substrate, however, no activities have been detected. Surprisingly, when homo- and hetero-polymeric blocks derived from sodium alginate were used to screen the activity, a strong activity was observed for the homo- and hetero-polymeric blocks in the intracellular fraction. We report here on the preliminary characterization, including the mode of action, of the intracellular homo- and hetero-polymeric block degrading enzyme activity of *Alteromonas* sp. strain H-4, and on our finding that the intracellular enzymes scarcely degrade intact alginate and generate a dimeric form from polyM. We expect that the intracellular enzymes may play an important role in the degradation and metabolism of oligouronates generated by the extracellular enzyme action of the bacterium.

Materials and Methods

Bacterial Strain and Culture Medium

The alginolytic marine bacterium, *Alteromonas* sp. strain H-4 used in this study has been previously described.[3-5] The bacterium was maintained using AI 2 agar medium[3] at 20°C.

Preparation of Intracellular Enzyme

Alteromonas sp. strain H-4 was cultured in 450 ml of a seawater broth (pH 7.5) containing 0.5% casitone (Difco), 0.1% sodium alginate (Wako Pure Chemical Industries, Ltd; 300-400 cp) and 75% seawater at 25°C. The culture was centrifuged at 10,000 × *g* for 15 min, and the bacterial cells were collected. To prepare the intracellular enzyme, the cells were washed 3 times with artificial seawater (NaCl 30.0 g, $MgCl_2$ 10.8 g, KCl 0.7 g, $MgSO_4$ 5.3 g, $CaSO_4$ 1.3 g in 1,000 ml distilled water) containing 4 mM Tris (pH 7.8). The cells were resuspended in 5 volumes of 0.1 M Tris-HCl buffer (pH 7.5) per wet weight of the cells and frozen at −20°C overnight. The cell suspension was disrupted by sonication (150 W, 30 sec, 5 times) after thawing. Cell debris was removed by centrifugation (7,000 × *g*, 15 min,

4°C). The supernatant was used for crude intracellular enzyme preparation.

To characterize the enzyme activity, the enzyme was partially purified by two-step (50 and 90% saturated) ammonium-sulfate precipitation. The resultant precipitation was dissolved in 0.1 M Tris-HCl buffer (pH 7.5) and dialyzed in 0.1 M Tris-HCl buffer (pH 7.5). The partially purified enzyme was stored at −20°C before use.

Substrate and Enzyme Assay

PolyM, polyG and MG random blocks were prepared from sodium alginate according to Haug *et al.*[6] The uronate composition was quantified using H^1 NMR[7,8] and presented in a previous report.[3] Mannuronate lactone was purchased from Sigma. The unsaturated oligouronates generated from sodium alginate by *Alteromonas* extracellular alginate lyase were separated by GCL 25 m (Saikagaku Kogyo, Co.) gel filtration chromatography using distilled water as an eluent. The active fractions were then pooled and lyophilized.

The thus-prepared intracellular enzyme solution was added to a 0.1% substrate solution in 0.1 M Tris-HCl buffer (pH 7.5). The enzyme activity was monitored by measuring the increase in absorbance at 235 nm using a Hitachi spectrophotometer (Type 124) from substrates containing 0.1% sodium alginate, homo- and hetero-polymeric blocks, mannuronate lactone or the lyophilized unsaturated oligouronate. One enzyme unit was defined as an increase in optical density (235 nm) of 0.010 per min.[4,5] Alternatively, enzyme activity was also measured by Somogy-Nelson's reducing sugar determination[9] using sodium alginate or homo- and hetero-polymeric blocks as the substrate.

Enzyme Activity on Gels

The intracellular fractions were prepared at each growth phase (early log phase: 48 h incubation, late log phase: 72 h, stationary phase: 96 h, and decreasing phase: 120 h) of the bacterium. Degrading activities of crude preparation of the intracellular fraction for Alg-Na and homo- and hetero-polymeric blocks were investigated using the activity staining technique according to our previous report.[3]

Characterization of the Enzyme Activity

The effect of pH on the partially purified enzyme activity for polyM block was determined by measuring the increase in absorbance at 235 nm in a broad pH range buffer[10] containing 0.1 M Tris, 0.1 M acetic acid, 0.1 M MOPS, 0.1 M MES and 0.1 M glycine at adjusted pH levels of 4 to 10 at 35°C. The effect of temperature on the activity was measured at temperatures ranging from 10 to 50°C in a 0.1 M Tris-HCl buffer adjusted to pH 7.5.

Analysis of Products from Depolymerization of PolyM Block by the Intracellular PolyM Specific Lyase

Substrate solutions containing 6.5 mg/ml polyM block in 0.1 M Tris-HCl buffer (pH 7.5) were incubated for 24 h at 35°C with the partially purified intracellular enzyme. No further increase in absorbance at 235 nm was detected after this period. The reacted mixture separated and the reaction products were detected and analyzed by the same manner of the previous report.[3]

Results

Production of Intracellular Homo- and Hetero-polymeric Degrading Enzyme Activity from Alteromonas *sp. Strain H-4*

The intracellular enzyme production profiles of *Alteromonas* sp. H-4 are shown in Fig. 1. The activities of intracellular enzyme preparation for polyM block and MG random block were higher than those for polyG block and Alg-Na. The activities for polyG block and Alg-Na were half and one fifth of that for MG random block, respectively. The enzyme activities for polyM block and MG random block were highest when the maximum growth of the strain occurred (at 96 h cultivation).

The intracellular enzyme activity was also detected in the bacterial cells incubated in the seawater broth without sodium alginate (Table 1). The activity for polyM block (980 U/g wet cells) was approximately the same level as the activity from the alginate-deficient medium (820 U/g wet cells).

Activity Staining of the Intracellular Fraction on Gel

Two kinds of degrading spots for polyM, a degrading spot for MG block, and three degrading spots for polyG were detected from the intracellular fraction at each growing stage of the bacterium (Fig. 2). Trace spot was also detected for Alg-Na (Fig. 2). The p*I* value of the degrading spots for polyM blook was 5.4 and 6.7, respectively. The spot with p*I* 6.7 corresponded to the MG block degrading spot. A spot with p*I* 6.7 for polyG blook was only detected at the middle log phase and decreasing phase. Furthermore, other spots with p*I* 7.5 and 7.1 were detected at the late log phase to stationary phase, respectively.

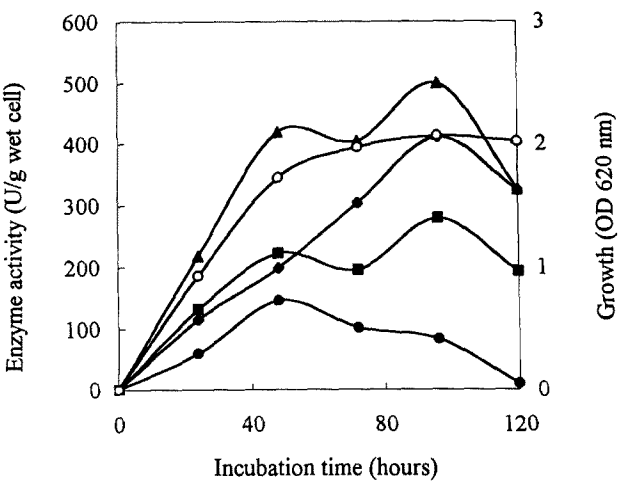

Fig. 1. Alginate degrading enzyme activity and the substrate specificity of intracellular preparation of *Alteromonas* sp. H-4. Intracellular fraction was prepared at each incubation time of *Alteromonas* sp. H-4 and its enzyme activity was measured at 30°C for sodium alginate (●), polyM block (◆), polyG block (■) and MG random block(▲) dissolved in 0.1 M Tris-HCl buffer (pH 7.5) as substrate. Growth of the strain was simultaneously measured at absorbance of 620 nm (○).

Table 1. Effect of sodium alginate addition into the culture medium on the production of the intracellular and extracellular enzyme activities for polyM block of *Alteromonas* sp. H-4

Medium	Activity for polyM block
Intracellular activity	(U/g cells wt)
Sodium alginate (0.1%)	980
Without sodium alginate	820
Extracellular activity	(U/m*l*)
Sodium alginate (0.1%)	5.1
Without sodium alginate	ND*

* Not detected

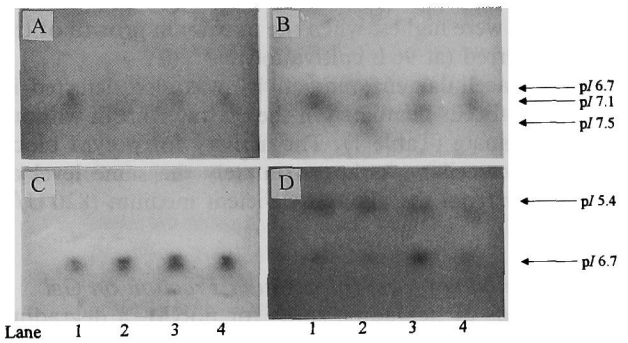

Fig. 2. Activity staining profiles of the intracellular enzymes of *Alteromonas* sp. H-4 for sodium alginate and homo- and hetero-polymeric blocks after isoelectric focusing gel electrophoresis. (A): sodium alginate, (B): polyG, (C): MG random and (D): polyM blocks. Lane 1: cell-bound activities at 48 h incubation, lane 2: at 72 h incubation, lane 3: at 96 h incubation, lane 4: 120 h incubation.

Estimation of Cleavage Pattern for PolyM Block by the Intracellular Enzyme Activity

The optimum temperature of the partially purified enzyme activity for polyM blook was 35°C and the activity was greatest at pH 7.0 to 7.5. To confirm whether the intracellular enzyme degraded the glycosidic linkage in polyM block or not, the activity was measured by reducing power determination and the UV absorption method under the optimum reaction condition. The results are shown in Fig. 3. The release of reducing sugar from polyM and MG random blocks by the enzyme degradation apparently increased. However, using MG random block as a substrate, no further increase of absorbance and reducing sugar release were observed at the end of the reaction periods, although the absorbance at 235 nm and the release of reducing sugar from the reaction mixture increased after 10 minutes of incubation. There was no release of reducing sugar from sodium alginate by the enzyme action.

The end products generated from polyM block by the cell-bound enzyme were separated by Cellulofine GCL 25 m gel filtration chromatography, and the elution profiles of unsaturated oligouronates are shown in Fig. 4. A strong absorption peak at 235 nm, which was not detected in the polyM block (Fig. 4. A-S), was observed around the void volume of the column after the enzyme action (Fig. 4. A-Digest). The corresponding peak contained considerable amounts of uronate (Fig. 4. B-Digest). Although a uronate peak was detected in the polyM block used as substrate at the same elution point (Fig. 4. B-S), the uronate content of

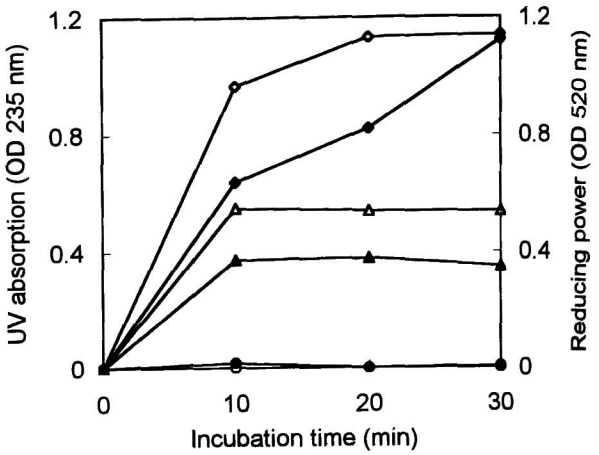

Fig. 3. Measurement of the partially purified intracellular activity for polyM block by UV absorption (open symbol) and reducing power determination (closed symbol). Activity of the partially purified cell-bound enzyme was measured at 30°C for sodium alginate (○ and ●), polyM block (◇ and ◆) and MG random block (△ and ▲) dissolved in 0.1 M Tris-HCl buffer (pH 7.5) as a substrate.

the peak decreased by half (Table 2). The unsaturated polysaccharides contained within the peak were estimated to have DP 23 (Table 2). This value is higher than the molecular weight (4,000), which is the exclusion limit of Cellulofine GCL 25 m as sugar. Four minor UV-absorption peaks were also detected (Fig. 4. A-Digest), but the uronate contents of the corresponding peaks were low (Fig. 4. B-Digest). Another uronate peak (peak I in Fig. 4.B-Digest) was observed after the UV-absorption peak (Fig. 4.A-Digest). This uronate peak did not show UV absorption and contained a high amount of reducing residues (Table 2). The DP of the saturated uronate was estimated to be 2, and 1.8 mg of uronates was converted to the dimeric form from 6.5 mg (equivalent to uronate) of polyM block by the enzyme action (Table 2). These results show that 4.7 μM saturated dimeric form and 0.7 μM unsaturated polyuronate (DP 23) are generated from polyM block.

Discussion

In addition to the production of an extracellular, nonspecific alginate lyase,[3] the marine bacterium *Alteromonas* sp. H-4 produces intracellular degrading activity only for homo- and hetero-polymeric blocks derived from alginate. After measuring the intracellular enzyme activity using the two methods, it was apparent that the intracellular enzyme activity is limited for intact alginate (Figs. 1, 2 and 3). The enzyme activity was strong for MG and polyM, but the enzyme activity for MG quickly reached a plateau (Fig. 3). Using polyM block, the partial characterization of the intracellular enzymes, which contained 4 kinds of enzymes, generates a saturated diuronate and unsaturated polyuronate with a longer glycosidic chains (Fig. 4 and Table 2). The molar ratio of saturated diuronate and unsaturated polyuronates was estimated to be 4.7 μM (DP=2) and 0.70 μM (DP=23). It appears that the intracellular enzymes may have hydrolytic cleavage action. However, the intracellular enzyme preparation contained at least 4 kinds

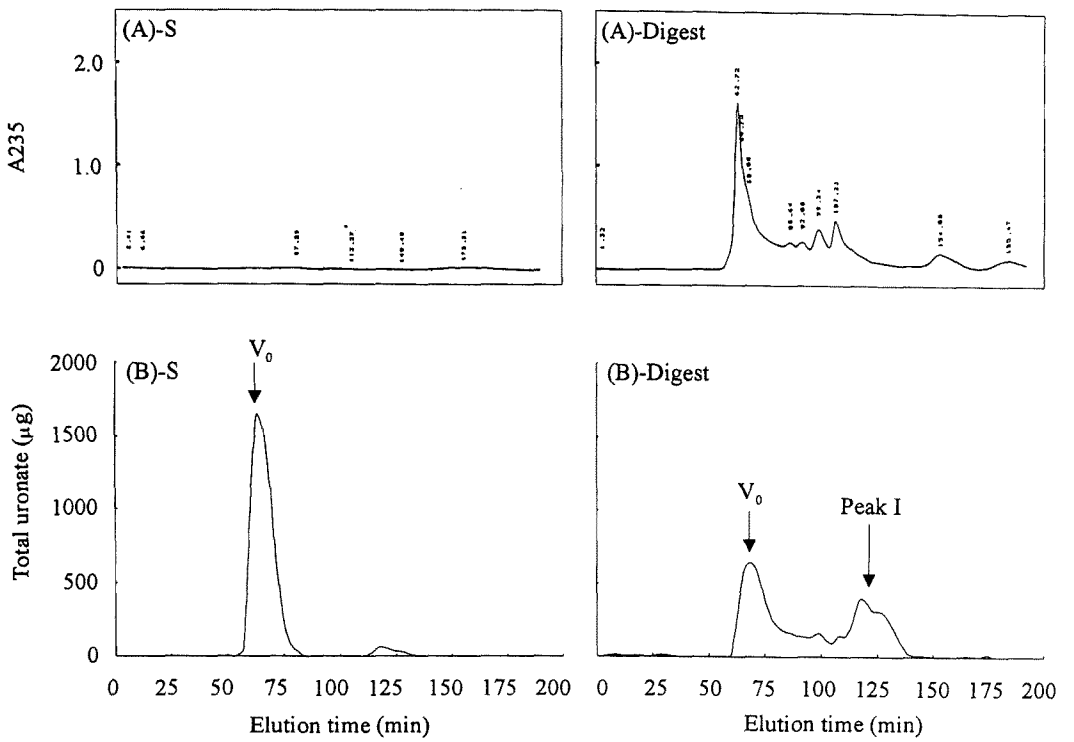

Fig. 4. Elution profiles of the reaction products from polyM block generated by the intracellular polyM specific lyase degradation. Substrate solutions containing 6.5 mg/ml polyM block in 0.1 M Tris-HCl buffer (pH 7.5) were incubated for 24 h at 35°C with the partial purified polyM specific lyase of *Alteromonas* sp. H-4. The undigested polyM block and reaction mixture was applied to a column (1.34 cm × 40 cm) of Cellulofine GLC 25 m and eluted with 0.1 M Na_2SO_4 at 28 ml/h. The undigested polyM block ((A)-S) and reaction products ((A)-Digest) were detected at 235 nm using a Hitachi L-4200 UV-VIS detector. The total amounts of uronate of the peaks were determined after separation of undigested polyM block ((B)-S) and reaction products ((B)-Digest) by gel-filtration chromatography. V_0 indicates void volume of the column.

Table 2. Analysis of reaction products from polyM block generated by the enzymes

Peak	Total amounts in a corresponding peak (mg)		Estimated DP of reaction product*2
	Uronate*1	Reducing termini	
Peak V_0	3.2 (0.7 μM)	0.12	22.9
Peak I	1.8 (4.7 μM)	0.66	2.2

*1 Parenthesis indicates molar ratio of oligouronates containing in each peak.
*2 Molar ratio of reducing termini was determined using glucose as standard and that of uronate was determined using polyM blcok as standard.DP value was caluculated from a ratio for uronate to reducing termini.

of enzymes with different substrate specificity and p*I*. It is not clear whether the generation of the saturated diuronate may be attributable to one of the enzymes in the intracellular preparation or to the combined action of all enzymes.

There have been no reports of intracellular enzymes that are incapable of degrading intact sodium alginate among all described alginate lyases.[1,2] Despite the fact that the intracellular enzyme of *Alteromonas* was incapable of degrading sodium alginate, the absorbance of the unsaturated oligouronate solution prepared from alginate digests by the *Alteromonas* extracellular alginate lyase doubled after the intracellular enzyme activity (data not shown). These observations in this study suggested that the intracellular enzymes may be specific for oligosaccharides. Many aspects of the mode of enzyme action remain unresolved. In future studies, we plan to homogeneously purify the enzymes and examine then in detail.

Among intracellular alginate lyases reported in *Klebsiella aerogenes* (type 25),[11] *Pseudomonas* spp.,[12-14] *Sphingomonas* sp.,[15] some marine pseudomonad,[16,17] *Beneckea pelagia*,[14] *Azotobacter* spp.,[18] and *Alteromonas* sp.,[19] a few intracellular enzymes were suggested to play a role in the final digestion process of alginate metabolism by bacteria. As Romeo *et al.*[19] proposed in marine *Alteromonas* sp, a high molecular alginate would be degraded to oligosaccharides by an extracellular, endo-acting enzyme of the bacterium, and the oligosaccharides would then be degraded to monomers by an intracellular exo-acting enzyme, and metabolized. *Alteromonas* sp. strain H-4 is capable of utilizing alginate as its sole carbon source.[4] High molecular alginates can be degraded by the extracellular enzyme to shorter-chained oligouronates, and the oligouronates may then be degraded to the shorter, dimeric form, by the intracellular enzymes. Regarding alginate metabolism by *Alteromonas*, the production of extracellular alginate lyase with broad substrate specificity and intracellular enzyme with oligouronate specificity are reasonable, although details of the metabolic pathway of alginate remain unsolved. The intracellular enzyme activity was also constitutively expressed in the bacterium similar to the extracellular alginate lyase.[4] The relationship between

the extracellular alginate lyase showing non-limited substrate specificity and the intracellular polyM block specific lyase in the alginate metabolism of *Alteromonas* sp. strain H-4 must be characterized.

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