

海洋性Alteromonas sp.の産生するアルギン酸リアーゼの精製と性状

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Purification and Characterization of an Alginate Lyase from Marine *Alteromonas* sp.

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An alginate lyase from the culture medium of *Alteromonas* sp. H-4 was purified by ultrafiltration, gel filtration, and anion-exchange chromatography and was characterized. A molecular weight of the purified enzyme was estimated as 32,000 by sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE). Optimum pH and temperature of the enzyme activity were 7.5 and 30°C, respectively. The enzyme was unstable on heating and in acidic solution. The alginate lyase required 50 mM MgCl₂ or MgSO₄, 0.5 M NaCl, and 0.2 M KCl for the maximum activity.

Alginate is the main composition in cell walls of brown algae and is utilized in food and medicine as mainly sodium alginate. It is a linear uronic acid polymer composed of β -D-mannuronic acid and α -L-guluronic acid in homopolymeric and heteropolymeric blocks. Depolymerizing enzymes of alginate have been detected from marine molluscs,¹⁻⁵⁾ marine⁶⁻¹⁰⁾ and terrestrial¹¹⁻¹⁵⁾ bacteria, marine fungi,¹⁶⁾ and brown algae.¹⁷⁾ These enzymes have been used to examine the structure of alginate and the composition of cell walls of brown algae.^{18,19)} These enzymes are also utilized for making protoplasts of marine brown algae.^{20,21)} Alginate lyases used in these studies were almost extracted from marine molluscs. However, reports on the utilization of bacterial alginate lyase are very limited.

The *Alteromonas* sp. strain H-4 used in this study was isolated from decaying thalli of *Laminaria* sp. and suggested as one of the causative agent of "Anaaki-sho" on Rishiri-konbu *Laminaria japonica* var. *ochotensis*.²²⁾ We describe here purification and some properties of the enzyme.

Materials and Methods.

Bacterium and Culture Medium

Alteromonas sp. H-4 isolated from decaying thalli of *Laminaria japonica* var. *ochotensis* was grown in a medium containing 0.8% casitone, 0.1% sodium alginate, 75% seawater as described previously.²²⁾

Enzyme Purification

Alteromonas sp. H-4 was grown at 25°C for 4 days in 500 ml flask containing 450 ml of the above medium with reciprocal agitation. The culture medium was centrifuged at 20,000×g for 30 min and the supernatant was used as the starting material for purification of the enzyme. All ensuing operations were carried out at 0-4°C.

The culture supernatant was concentrated with tangential flow filtration using a Millipore Pellicon cassette system with polysulfone membrane (PTCG) which allowed retention of proteins larger than 10 kDa, and then was dialyzed overnight against 0.1 M Tris-HCl buffer (pH 7.5). Insoluble materials were removed by centrifugation at 7,000×g for 20 min. The supernatant was concentrated by lyophilization and was subjected to gel filtration chromatography on a Sephadex G-100 column (2.6 cm×64 cm) in 0.1 M Tris-HCl buffer containing 50 mM MgCl₂ (pH 7.5), as eluant. Fractions showing alginate lyase activity were pooled and further fractionated on a DEAE-Sephadex A-50 column (2.6 cm×40 cm) equilibrated with 0.1 M Tris-HCl buffer (pH 7.5). After application of the enzyme fractions, the column was washed with the same buffer and then eluted with a linear gradient of NaCl (0-0.5 M) in 0.1 M Tris-HCl buffer (pH 7.5). Fractions with alginate lyase activity were collected and dialyzed against 0.1 M Tris-HCl buffer (pH 7.5). The fractions were lyophilized and subjected to a Sephadex G-75 column (2.6×94 cm) and then eluted as above Sephadex G-100. Active fractions were recovered, and concentrated, and

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subjected to gel filtration using a Toyopearl HW-50 fine grade (TOSOH Co. Ltd.) column (2.6 × 94 cm) and was eluted as above Sephadex G-100. Enzymatically active fractions from the Toyopearl gel were rechromatographed on the DEAE-Sephadex A-50 column as described above and eluted in the same manner with an NaCl gradient (0–0.7 M). The enzyme solution was stored at –20°C until use.

Enzyme Assay

Quantitative estimates of alginate lyase were made by measuring the increase in absorbance at 235 nm with a Hitachi spectrophotometer.^{1,5)} For the enzyme reactions, unless otherwise noted, a reaction mixture contained 0.1% sodium alginate, 0.1 M Tris-HCl (pH 7.5), and was incubated for 10 min at 30°C. One unit of enzyme activity was defined as an increase in optical density (235 nm) of 0.010 per min.

In some cases, the enzyme activity was also measured by the method of Somogyi and Nelson's reducing sugar determination²³⁾ and viscometric assay with an Ostwald's viscometer.²⁴⁾

Protein contents in fractions at various stages of purification were determined by using an improved protein-dye binding assay.²⁵⁾

Characterization of Alginate Lyase

The effect of pH on the activity was measured using acetate buffer (pH 4.0–6.0), phosphate buffer (pH 6.0–7.0), and Tris-HCl buffer (pH 7.0–9.0) at 30°C. To investigate pH-stability, purified enzyme (0.1 ml) was added to the above various pH buffer (0.9 ml) and the mixtures were stored at 0°C for 6 h prior to assay.

The effect of temperature on the activity was measured at temperatures ranging from 10 to 60°C in Tris-HCl buffer (pH 7.5). Heating

stability of the enzyme was measured as follows: portions of purified enzyme were heated for 10 min in sealed tubes at 30 to 70°C, cooled in an ice bath, and were assayed for activity as mentioned above.

The effects of various salts, metals, and chemical compounds on enzyme activity were assayed in reaction mixtures containing 0.1% sodium alginate in 0.1 M Tris-HCl (pH 7.5), and one of the following compounds: MgCl₂, MgSO₄, NaCl, KCl, KH₂PO₄, and Na₂HPO₄ as salts; ethylenediamine tetraacetic acid trisodium salt (EDTA·3Na) and dithiothreitol (DTT) as chemical compounds; MnCl₂, CoCl₂, NiCl₂, ZnSO₄, CdCl₂, and BaCl₂ as metals. The final concentration of each compounds in the reaction mixture was 1 mM.

Electrophoresis

The purified enzyme was dialyzed against Tris-HCl (pH 7.5) and lyophilized and was dissolved in 20 μl of a solution containing 7% glycerol, 2% sodium dodecyl sulfate (SDS), 5% mercaptoethanol, 0.0625 M Tris, and 0.005% bromophenol blue (pH 6.8). This sample (9.0 μg of protein) was subjected to SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis was performed by using single-dimension 1.0 mm-thick slab gels of 10.0% acrylamide/0.27% bis-acrylamide (pH 8.8), for the running gel and 2.5% acrylamide/0.21% bis-acrylamide (pH 6.8), for the stacking gel.²⁶⁾ After electrophoresis, the gel slabs were stained for 1 h with Coomassie blue R-250. Bovine albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.2 kDa were used as

Table 1. Purification of alginate lyase from *Alteromonas* sp. H-4

	Volume (ml)	Activity* ¹ (U/ml)	Protein* ² (μg/ml)	Specific activity (U/μg)	Folds* ³	Yield (%)
Culture medium	4916.0	4.28				100
Ultra filtration	356.0	28.7	142.5	0.20	1.0	48.5
Sephadex G-100	333.8	22.1	12.3	1.80	8.9	35.1
DEAE Sephadex A-50	135.8	32.5	14.1	2.30	11.5	21.0
Sephadex G-75	145.5	28.5	7.94	3.59	17.9	19.7
Toyopearl HW-50F	34.6	96.7	25.9	3.73	18.7	15.9
2nd DEAE Sephadex	44.2	37.5	11.6	3.23	16.2	7.87

*¹ Unit of alginate lyase activity was measured by UV assay.

*² Estimated by protein-dye binding assay with bovine serum albumin as standard.

*³ Calculated relative to specific activity of the ultra-filtrated fractions.

molecular weight markers.

Results

Purification of Alginate Lyase

Purification procedures of the enzyme were summarized in Table 1. The final yield and specific activity of the purified enzyme were 7.87% and 16.2 folds, respectively.

On the first step in purification of the enzyme from crude extract, Sephadex G-100 gel filtration afforded a 9-fold purification. Fractions having alginate lyase activity were pooled and further fractionated on DEAE-Sephadex A-50. The enzyme was eluted in about 0.3 M NaCl. A peak of protein became single but did not correspond completely with an enzyme active peak. Further fractionations by gel filtration on Sephadex G-75 and Toyopearl HW-50F were performed. On Toyopearl HW-50F gel filtration both peaks of alginate lyase activity and the protein content coincided. To ascertain purification, DEAE-Sephadex chromatography was reoperated (Fig. 1). Although specific activity decreased, both peaks of the activity and protein content completely coincided. SDS-PAGE of the protein at a final step on the purification showed a single band estimated as 32 kDa (Fig. 2).

Characteristics of the Purified Enzyme

The purified alginate lyase had the activity at pH ranging from 6.0 to 8.0 and showed the maximum activity at pH 7.6 (Fig. 3-A). The optimum temperature for the enzyme activity was 30°C (Fig. 3-B). At 20 and 40°C, more than 60% of maximum activity was observed, but no activity was found at 60°C.

The pH and heat stability on the activity of the enzyme are shown in Fig. 4. The enzyme was very stable at pH values ranging from 6.6 to 9.0. However, the activity decreased to less than 20% at pH below 5.0 (Fig. 4-A). Treatment of the enzyme at 30 and 40°C for 5 min caused 20% and 40% decrease in the enzyme activities, respectively (Fig. 4-B).

Effects of various salts on the enzyme activity are shown in Fig. 5. The enzyme was stimulated by the addition of 50–100 mM $MgCl_2$ or $MgSO_4$ and the relative activity was two-folds higher than the control. Activities were also gradually increased by the addition of NaCl and KCl up to 0.5 and 0.2 M, respectively. The enzyme activity was inhibited by the addition of more than 0.1 M Na_2HPO_4 and 0.01 M KH_2HPO_4 .

The effects of chemical compounds and metals on the activity are shown in Table 2. Alginate lyase activity was stimulated by the addition of $MnCl_2$ or $BaCl_2$, but was inhibited by EDTA·3Na, $ZnSO_4$, or $CdCl_2$.

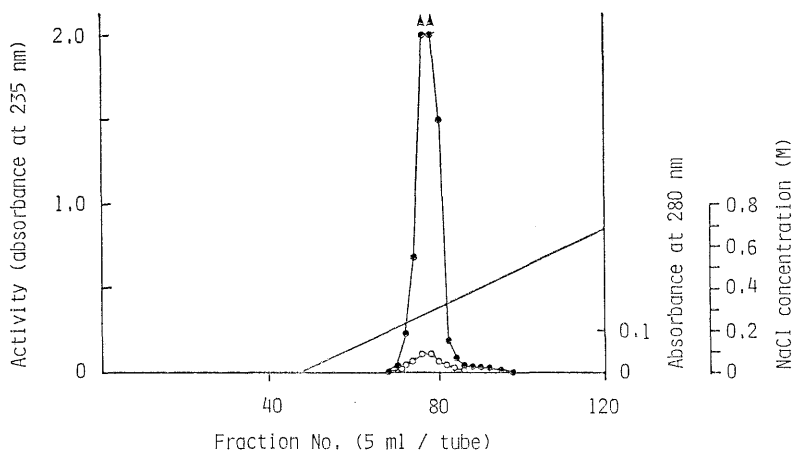


Fig. 1. Re-chromatography of alginate lyase on DEAE-Sephadex A-50.

Concentrated enzyme after Toyopearl gel filtration was applied on DEAE-Sephadex A-50 column (2.6 cm × 40 cm) and eluted with 0 to 0.7 M NaCl gradient. The flow rate was 25 ml/h at 4°C.

- (●), alginate lyase activity.
- (○), protein.
- (▲), more than detectable limit.
- Solid line indicates NaCl gradient.

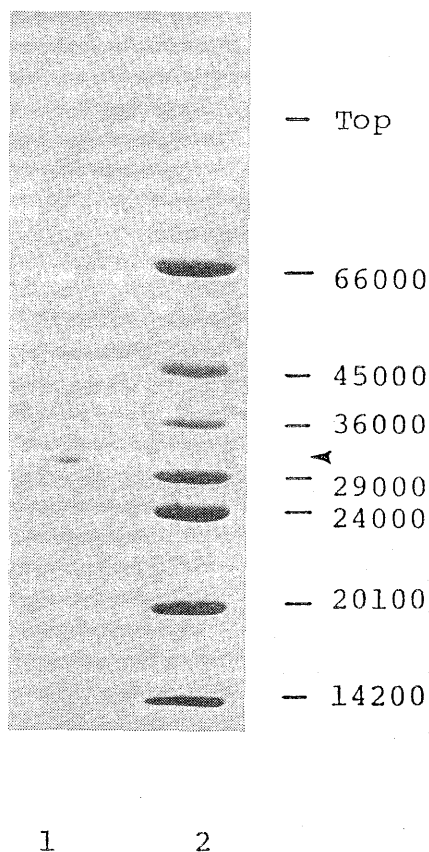


Fig. 2. SDS-polyacrylamide gel electrophoresis of purified alginate lyase from *Alteromonas* sp. H-4. Lane 1: alginate lyase (6 μ g). Lane 2: bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphite dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20.1 kDa; α -lactalbumin, 14.2 kDa.

Reaction Profile of Purified Alginate Lyase

Activities of the purified alginate lyase measured by the methods of ultraviolet absorption, reducing sugar determination and viscometric assay are shown in Fig. 6. With the enzyme reaction to sodium alginate, production of substances with absorption at 235 nm was parallel with the increase of reducing sugar, on the other hand, the viscosity of the substrate immediately decreased for the first 2 min by the purified alginate lyase and slightly decreased thereafter.

Discussion

A few of alginate lyases have been homogeneously purified from invertebrates⁵⁾ and marine *Photobacterium*,¹⁰⁾ but others have been examined in partially purified states from marine and terrestrial bacteria.^{4,14,27)} Preston *et al.*²⁷⁾ reported that *Alteromonas* sp. isolated from seaweed produced an extracellular alginate lyase, of which specificity was estimated using unpurified enzyme. In the present study, we succeeded in purifying alginate lyase of *Alteromonas* sp. H-4.

Through ultrafiltration, gel filtration with Sephadex G-100 and G-75, Toyopearl HW-50F, and anion-exchange chromatography using DEAE Sephadex A-50, purification of the alginate lyase was attempted and homogeneity of the purified enzyme was confirmed on SDS-PAGE (Fig. 2). On the first gel filtration with Sephadex G-100, at least 90% of total protein was excluded (Table 1), and recovery of alginate lyase activity was the highest by repeated gel filtration chromatography. The gel filtration chromatography was effective

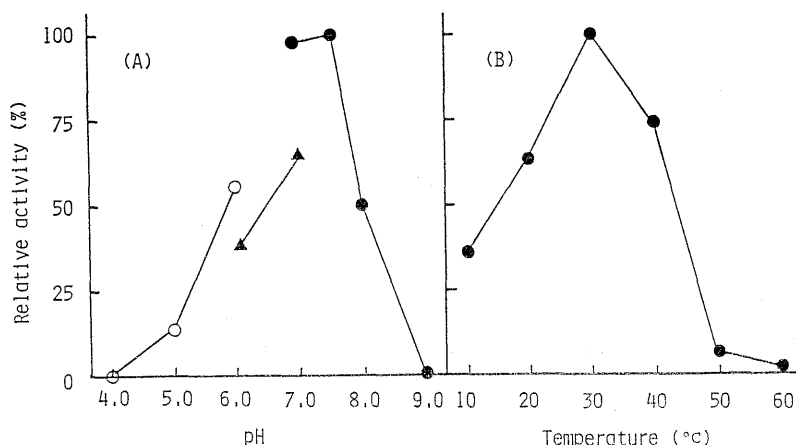


Fig. 3. Effects of pH (A) and temperature (B) on alginate lyase activity. (A): (○) 0.1 M acetate buffer, (▲) M/30 phosphate buffer, (●) 0.1 M Tris-HCl buffer.

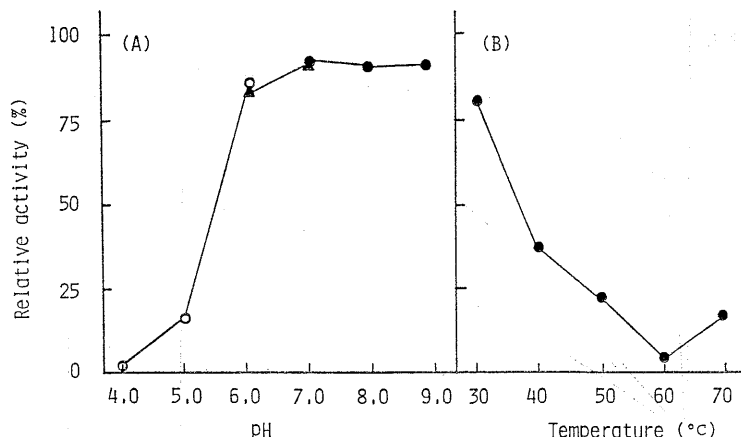


Fig. 4. Effects of pH (A) and heating (B) on the stability of alginate lyase.
(A): (○) 0.1 M acetate buffer, (▲) M/30 phosphate buffer, (●) 0.1 M Tris-HCl buffer.

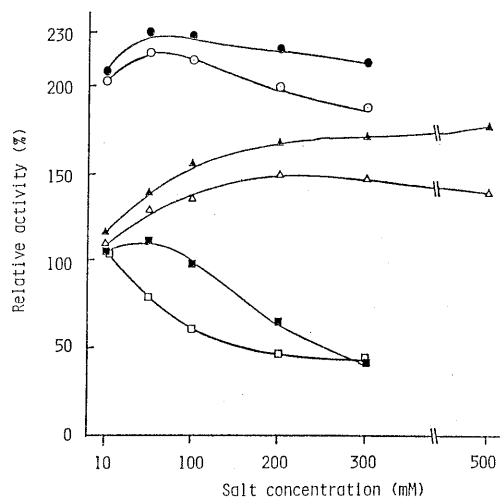


Fig. 5. Effects of various salts and salt concentrations on alginate lyase activity.

(●), $MgCl_2$; (○), $MgSO_4$; (▲), $NaCl$; (△), KCl ;
(■), Na_2HPO_4 ; (□), KH_2PO_4 .

on the purification of the alginate lyase.

The alginate lyase reaction caused the increases in UV absorption (235 nm) and reducing sugar in the reaction mixture (Fig. 6). Tujino and Saito²⁶⁾ reported that unsaturated diuronide with absorption at 235 nm was isolated as the reaction product in the reaction mixture of alginate lyase from abalone intestine. Furthermore, formation of reducing sugar was parallel with that of UV absorption materials. It was suggested that the alginate lyase of *Aeromonas* sp. H-4 was not hydase such as cellulase or chitinase, but that the enzyme cleaved alginate resulting in the

Table 2. Effects of compounds and metals on alginate lyase activity

Compound* ¹	Relative activity (%)
EDTA·3Na* ²	32.5
DDT* ³	94.2
$MnCl_2$	138.3
$CoCl_2$	89.7
$NiCl_2$	86.1
$ZnSO_4$	49.7
$CdCl_2$	30.8
$BaCl_2$	143.1
control	100.0

*¹ All compounds were added at 1 mM each to enzyme mixture.

*² Ethylenediamine tetraacetic acid, trisodium salt.

*³ Dithiothreitol.

formation of series of oligosaccharides containing unsaturated residues on the non-reducing end of the glycoside chain and was the same as alginate lyase EC [4.2.2.3] from other sources.^{1,26)} Moreover, the alginate lyase remarkably decreased the viscosity of the substrate and from the result, it is speculated that the enzyme was endo-type alginate lyase.

Kitamikado *et al.*²⁴⁾ reported isolation of two alginolytic *Vibrio*, strain AI-9 and AI-128, from seaweed and fish intestine, respectively. The reaction profile of alginate lyase from the strain AI-9 was strong to produce reducing sugar, and that of the strain AI-128 was strong to decrease viscosity of substrate. On the other hand, the enzyme from *Alteromonas* sp. strain H-4 has both characters of reaction profiles. The optimum pH and salt requirement of this enzyme

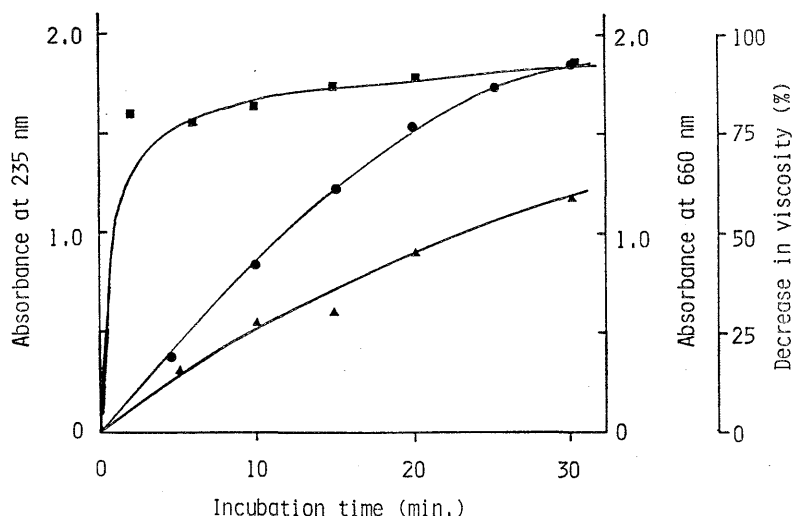


Fig. 6. Reaction profiles of purified alginate lyase measured by the method of UV absorption, reducing sugar and viscometric assay.

- (●): UV absorption at 235 nm.
 (▲): reducing sugar.
 (■): viscosity.

were almost the same as enzymes from other sources except terrestrial bacterium. From the above results, we concluded that the alginate lyase of *Alteromonas* sp. H-4 might be very active in seawater and adapted to the marine environment. It is expected that this enzyme is able to effectively degrade alginate in the cell walls of brown algae and may likewise serve to make protoplasts.

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