

Vibrio alginolyticus ATCC 17749の産生するアルギン酸リアーゼ

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Alginate Lyase from *Vibrio alginolyticus* ATCC 17749

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Vibrio alginolyticus ATCC 17749 was proved to produce an extracellular alginate lyase. The enzyme was purified from the culture fluid by ammonium sulfate precipitation followed by phenyl Sepharose CL-4B and blue Sepharose CL-6B chromatographies. The final enzyme preparation was homogeneous as judged by SDS-polyacrylamide gel electrophoresis. The lyase was found to be specific for β -1,4 bonds involving D-mannuronate units in alginate. A calcium chloride concentration of 1×10^{-2} M activated the enzyme to reach a maximal activity, and calcium chloride was also effective on the increase in thermostability of the enzyme.

We tried to obtain two types of alginate lyase, [poly(1,4- α -L-guluronide) lyase and poly(1,4- β -D-mannuronide) lyase], from bacterial origins. Two poly(1,4- α -L-guluronide) lyases have recently been isolated from the culture fluids of *Vibrio harveyi* AL-128¹⁾ and *V. alginolyticus* AL-9.²⁾ On the other hand, a poly(1,4- β -D-mannuronide) lyase was isolated from the culture fluid of *V. alginolyticus* AL-9, and some complex procedures were necessary to purify the enzyme.²⁾ Therefore, another bacterial source for obtaining the enzyme is needed.

According to Bergey's Manual of Systematic Bacteriology,³⁾ members of the species *V. alginolyticus* are negative for the production of extracellular alginate-degrading enzyme. However, we found that the type strain of *V. alginolyticus* ATCC 17749 releases an extracellular alginate lyase into the culture fluid.⁴⁾ Since this alginate lyase was more active toward manuronate-rich substrate (M-blocks) than guluronate-rich substrate (G-blocks), the bacterium was expected to be useful for the preparation of a poly-1,4- β -D-mannuronide lyase. In the present paper, the isolation and partial characterization of this enzyme are described.

Materials and Methods

Organism

V. alginolyticus ATCC 17749 was obtained from the American Type Culture Collection. The organism was grown on a medium containing

0.5% peptone, 0.1% yeast extract, 3.0% sodium chloride, 0.5% sodium alginate, and 1.5% agar (pH 7.8), was stored at 4°C, and was transferred monthly on the medium to maintain its viability. The liquid medium used for culturing the organism contained 1.0% peptone, 0.1% yeast extract, 3.0% NaCl, and 0.5% sodium alginate (pH 7.8).

Materials

Sodium alginate was obtained from Wako Pure Chem. Ind. Ltd. and was used as the usual substrate throughout this experiment. G-blocks ($\overline{DP}=9$) and M-blocks ($\overline{DP}=13$) were prepared from sodium alginate by the method of Haug *et al.*⁵⁾ Phenyl Sepharose CL-4B and blue Sepharose CL-6B were obtained from Pharmacia LKB Biotechnol., Sweden. Peptone and yeast extract were obtained from Nihonseiyaku Ltd. All other chemicals were obtained from commercial sources.

Purification of Alginate Lyase

The cells taken from the slant culture were inoculated into 20 ml of the liquid medium in a 50 ml flash, and incubated without shaking at 25°C for 2 days. The culture was then transferred to 1,000 ml of the liquid medium in a 3,000 ml flask, and incubated without shaking at 25°C for 5 days. The culture fluid (2,690 ml) was obtained by centrifugation at $10,000 \times g$ for 30 min. This crude enzyme preparation was further purified by ammonium sulfate fractionation as well as phenyl Sepharose CL-4B and blue Se-

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pharose CL-6B chromatographies. All operations described below were carried out at 5°C.

1. Ammonium sulfate fractionation: The culture fluid of the strain (2,690 ml) was adjusted to 75% saturation with solid ammonium sulfate, and allowed to stand overnight. The precipitate formed was collected by centrifugation and dissolved in 200 ml of 10 mM sodium phosphate buffer (pH 6.8) for further purification.

2. Phenyl Sepharose CL-4B chromatography: A 50 ml portion of the above enzyme solution was loaded to a phenyl Sepharose CL-4B column (2.0×24 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.8) containing 1.8 M ammonium sulfate. After washing with 800 ml of the same buffer, the enzyme was eluted by 1,000 ml of phosphate buffer (pH 6.8) with a linear gradient of 1.8–0 M ammonium sulfate. The main active component (tube no. 166–174 in Fig. 1) was pooled, concentrated with polyethylene glycol, and dialyzed against 50 mM sodium acetate buffer (pH 6.0).

3. Blue Sepharose CL-6B chromatography: The dialyzed preparation was applied to a blue Sepharose CL-6B column (2.5×17 cm) equilibrated with 50 mM sodium acetate buffer (pH 6.0). After washing with 200 ml of the same buffer, the enzyme was eluted ascendingly by 300 ml of the buffer with a linear gradient of 0–1.0 M sodium chloride. The active component was pooled, concentrated with polyethylene glycol, and dialyzed against 50 mM Tris-HCl buffer (pH 8.0).

Enzyme Assays

1. Reducing group assay: To 1.5 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 0.4% sodium alginate and 0.4 M sodium chloride was added 0.5 ml of enzyme solution. After incubation at 37°C for 20 min, the reaction was stopped by adding 2.0 ml of the alkaline copper reagent of the Somogyi-Nelson method,⁶⁾ and the reducing sugar produced was determined. One unit of the enzyme is defined as the amount which liberated 1 μ mol of D-mannuronic acid or L-guluronic acid per min under the above conditions.

2. Thiobarbituric acid (TBA) assay: To 0.3 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 0.4% sodium alginate and 0.4 M sodium chloride was added 0.1 ml of the enzyme solution. After incubation at 37°C for 20 min, 0.5 ml of 0.025 N periodic acid in 0.125 N sulfuric acid was added to the reaction mixture. After 20 min at room temperature, 1.0 ml of 2.0% sodium arsenite

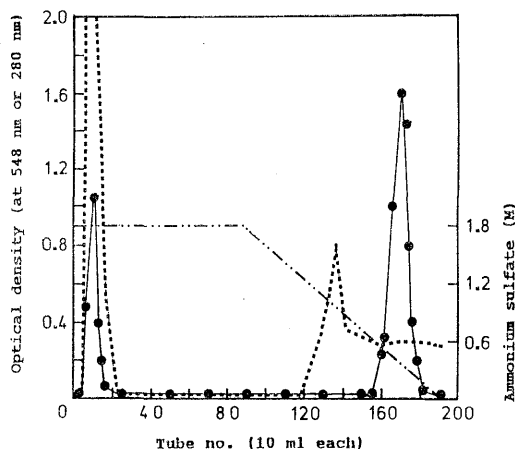


Fig. 1. Chromatography of alginate lyase from *Vibrio alginolyticus* ATCC 17749 on phenyl Sepharose CL-4B. ●—●, Enzyme activity (O.D. at 548 nm); ----, absorbance at 280 nm; ·····, concentration of ammonium sulfate.

in 0.5 N HCl was added with shaking, and the mixture was permitted to stand for 2 min. Four ml of 0.3% TBA were added and, after stirring, the mixture was heated at 100°C for 10 min. When the mixture had cooled, the optical density was measured at 548 nm.⁷⁾

SDS-Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide disc gel electrophoresis was performed on a 10% polyacrylamide gel containing 0.1% SDS at a constant current of 6mA per gel.⁸⁾ Proteins were stained with Coomassie brilliant blue R-250, and gel columns were destained in 7% acetic acid.

Results

Isolation of Alginate Lyase

The crude enzyme solution, after ammonium sulfate fractionation, was chromatographed on the phenyl Sepharose CL-4B column. As shown in Fig. 1, two enzymatically active peaks were evident. The main active component (tube nos. 166–174) was further purified by chromatography on the blue Sepharose CL-6B column. Only a single active peak was observed in Fig. 2. This fraction gave a single protein band when examined by SDS-polyacrylamide gel electrophoresis (Fig. 3) and was used as the purified alginate lyase preparation throughout this experiment.

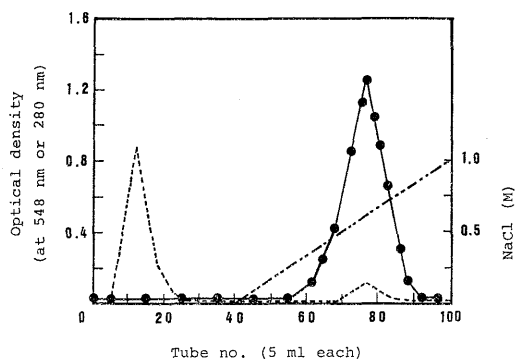


Fig. 2. Chromatography of alginate lyase on blue Sepharose CL-6B. ●—●, Enzyme activity (O.D. at 548 nm); ----, absorbance at 280 nm; ·····, concentration of sodium chloride.

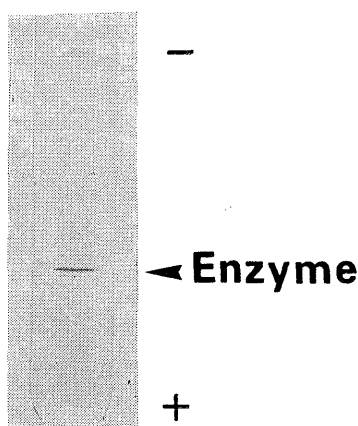


Fig. 3. SDS-PAGE of the final preparation of alginate lyase. (+), Anode; (-), cathode.

Effect of Calcium Chloride Concentration on Alginate Lyase Activity

The effect of calcium chloride on the enzyme activity was examined by incubating it in 50 mM Tris-HCl buffer (pH 8.0) containing 0.10 unit of enzyme, 0.4% sodium alginate (or 0.1% M-blocks), and 1×10^{-6} to 1 M calcium chloride. The enzyme activity reached a maximal level (33 times as high as the control) at a calcium chloride concentration of 1×10^{-2} M (Fig. 4).

Effect of Temperature on the Stability of Alginate Lyase

The enzyme (1.5×10^{-3} unit) was kept in 50 mM Tris-HCl buffer (pH 8.0) at various temperatures for 15 min. The remaining enzyme activity was then determined by the TBA method. The activity at 0°C was taken as 100%. On the

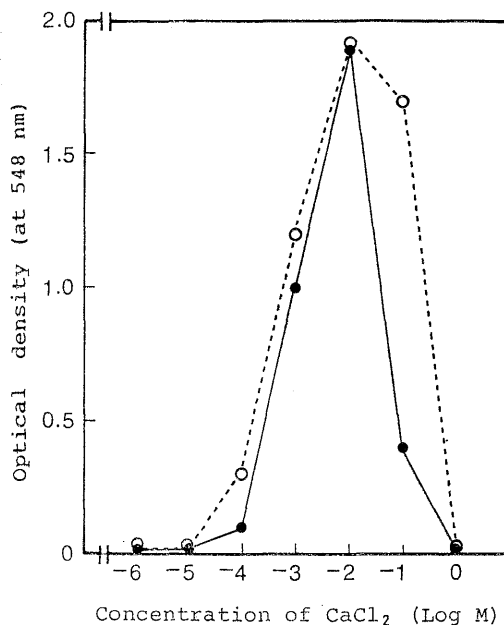


Fig. 4. Effect of calcium chloride concentration on the activity of alginate lyase. ●—●, Degradation of alginate; ○-----○, degradation of M-blocks.

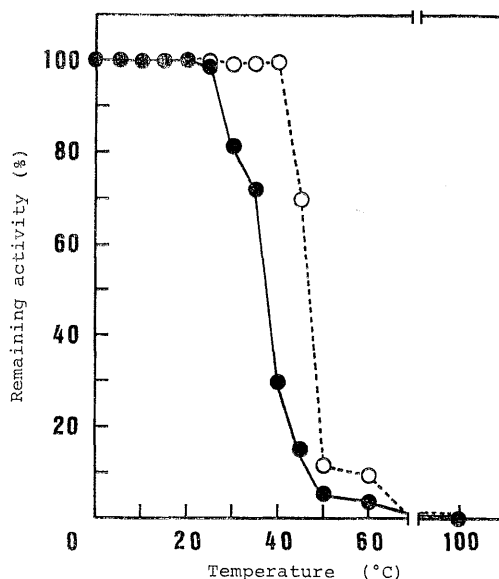


Fig. 5. Effect of temperature on the stability of alginate lyase. The enzyme solution was heated for 15 min at the designated temperature, cooled in iced water, and then assayed for activity. The activities obtained at 0°C were taken as 100%, respectively. ●—●, 50 mM Tris-HCl buffer, pH 8.0; ○---○, the same buffer containing 5 mM calcium chloride.

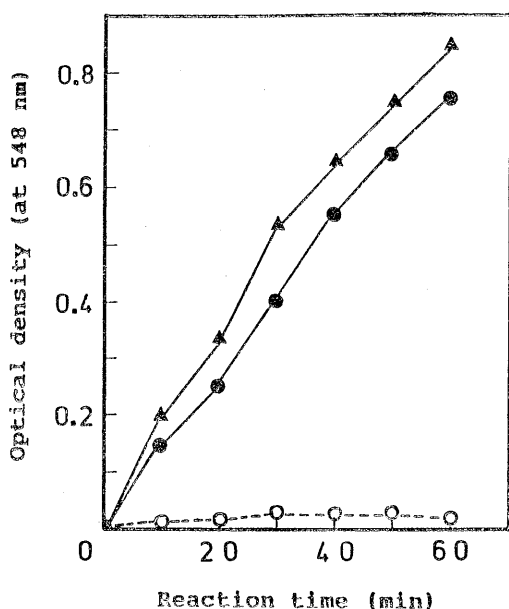


Fig. 6. Degradation of alginate, M-blocks, and G-blocks by alginate lyase. ▲—▲, Alginate; ●—●, M-blocks; ○—○, G-blocks.

other hand, the enzyme (1.5×10^{-3} unit) was kept in the same buffer containing 5 mM calcium chloride under the above conditions and the remaining enzyme activity was determined. The activity at 0°C in the presence of calcium chloride was taken as 100%. In the absence of calcium chloride the enzyme retained full activity at temperatures below 25°C , whereas in the presence of calcium chloride it was stable at temperatures up to 45°C (Fig. 5).

Substrate Specificity of Alginate Lyase

The enzyme solution of 2.0 ml (0.10 unit/ml) was incubated with 6.0 ml of each of 0.05% M-blocks, 0.05% G-blocks, and 0.4% sodium alginate solution, and the degradation of each substrate was determined at intervals of 10 min by the TBA method. As shown in Fig. 6, the degradation is recognized in M-blocks and alginate, but not in G-blocks.

Discussion

There were the plural components of alginate lyase in the culture of the strain ATCC 17749, and a main component adsorbed on the phenyl Sepharose CL-4B column was purified by the subsequent blue Sepharose CL-6B column chromatography to a homogeneous state. In this

study, the strain ATCC 17749 has been proved to be an efficient source of polymannuronide lyase.

Mannuronide lyases were reported to be obtained from marine molluscus,^{9,10} *Azotobacter vinelandii* phage,¹¹ and *Photobacterium* sp.¹² The lyases from *A. vinelandii* phage and *Photobacterium* sp. were activated (twice as high as the control) in the presence of 5×10^{-2} M calcium chloride. The enzyme from strain ATCC 17749 showed maximal activity (33 times as high as the control) at a calcium chloride concentration of 1×10^{-2} M. Under the presence of different substrates (alginate or M-blocks), however, a calcium chloride concentration of 0.1 M had different promoting effects toward the lyase from strain ATCC 17749 (Fig. 4). These results may be explained by the observation¹³ that the strong complex of poly-G-blocks in alginate and divalent ions caused a decrease in contact frequency between alginate molecule and enzyme. Other properties of this enzyme were described in our previous paper.¹⁴

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