Purification and Characterization of Alginate Lyase from Marine Vibrio sp. YWA

Yuan-Hong WANG*, Guang-Li YU, Xin-Min WANG, Zhi-Hua LV, Xia ZHAO, Zhi-Hong WU, and Wei-Shang JI
Marine Drug and Food Institute, Key Laboratory of Marine Drugs of Shandong Province, Key Laboratory of Marine Drugs, Ministry of Education, Ocean University of China, Qingdao 266003, China

Abstract Extracellular alginate lyase secreted by marine Vibrio sp. YWA, isolated from decayed Laminaria japonica, was purified by a combination of ammonium sulfate precipitation and diethylaminoethyl-Sephacel column chromatography. The results show that the molecular mass of alginate lyase was approximately 62.5 kDa, with an optimal pH and temperature at pH 7.0 and 25 ºC, respectively. \( K_m \) was approximately 72.73 g/L. The activity of the enzyme was enhanced by EDTA and Zn\(^{2+}\), but inhibited by Ba\(^{2+}\). The substrates specificity analysis shows that it was specific for hydrolyzing poly-\( \beta \)-1,4-mannuronate in alginate.

Key words marine Vibrio; alginate; alginate lyase; purification; characterization

Alginate is a linear polysaccharide composed of two monomers, \( \beta \)-D-1,4-mannuronate (M) and \( \alpha \)-L-1,4-guluronate (G), and arranged in three forms, poly \( \beta \)-D-1,4-mannuronate (M), poly \( \alpha \)-L-1,4-guluronate (G), and heteropolymeric random sequences (MG), [1]. Alginate is mainly produced by brown alga, and can also be secreted by some pathogenic bacteria, such as Pseudomonas aeruginosa which is known to be a component of the capsule-like biofilm responsible for chronic pulmonary infection and respiratory difficulty in patients with cystic fibrosis [2]. Alginate-derived oligosaccharides produced by alginate lyases have been shown as functional oligosaccharins [3], such as bifidus factor [4] and elicitor of plant growth [5].

In this paper, a marine bacteria (Vibrio sp. YWA), which could secrete alginate lyase, was screened and isolated from the decayed Laminaria japonica, and its biochemical property of the purified alginate lyase was reported.

Materials and Methods

Materials and instruments
Kelp (L. japonica) was collected from Qingdao seashore in May 2003 (Qingdao, China). Alginate (1%, 120 mPas) was purchased from Qingdao Algae Industry Company (Qingdao, China). (M) and (G) were prepared according to Wang et al. [13] and Liu et al. [14]. DEAE-Sephacel (90 μm) was from Amersham Pharmacia Biotech (Piscataway, USA). Biocard 700E perfusion chromatography (ABI Company, Foster City, USA), J2-MC centrifuger (Beckman, Fullerton, USA), Unico spectrophotometer (UV-2102 PCS; Dayton, USA), HPLC (Waters, Milford, USA) and standard protein marker (Promega, Madison, USA) were purchased respectively. Other reagents used were of analytical grade.

Screening and cultivation of the zymogenic strain
The rotted part of the kelp was cut and put into sterile seawater, and the suspension was diluted and added to

Received: April 10, 2006   Accepted: June 10, 2006
This work was supported by the grants from the National High Technology Research and Development Program of China (2002AA624020) and the Science and Technology Bureau of Qingdao (05-2-HY-49)
*Corresponding author: Tel, 86-532-82032064; Fax, 86-532-82033054; E-mail, yhwang@ouc.edu.cn

DOI: 10.1111/j.1745-7270.2006.00210.x
selective medium [10 g/L alginate, 30 g/L NaCl, 6 g/L K₂HPO₄, 3 g/L KH₂PO₄, 3 g/L (NH₄)₂SO₄, 0.5 g/L MgSO₄·7H₂O and 0.1 g/L FeSO₄·7H₂O] in which the alginate was used as the only carbon source. One week later, lines were drawn on the solid selective medium plate to culture the strains, and five generations were passaged successively. The solution was filtered for the second time after zymosis. The enzyme activity of each obtained strain was tested at 235 nm, and the strains were stored at −80 ºC. The Vibrio sp. YWA strain with the highest activity was cultured in the selective medium.

The strains were activated, and the monoclonal ones were transferred into a 500-ml erlenmayer flask containing 50 ml culture solution. The strains were agitated at 25 ºC for 12 h, and 2% of the total was transferred into a 500-ml erlenmayer flask containing 100 ml culture solution, with the same conditions described as above. The culture solution was collected after incubation for 48 h.

Isolation and purification of alginate lyase

The fermented culture solution was centrifuged for 30 min at 10,000 g, 4 ºC, and the supernatant was collected. (NH₄)₂SO₄ was added into the supernatant to a concentration of 35%, then the mixture was stirred for 2 h and centrifuged at 10,000 g for 20 min. The supernatant was further adjusted to 65% (NH₄)₂SO₄, stirred for 2 h at 4 ºC, and then centrifuged. The precipitate was collected and dialyzed against 20 mM phosphate buffer (pH 7.0) at 4 ºC for 24 h, and the crude enzyme solution was acquired.

The crude enzyme solution was applied to a DEAE-Sephacel column (1.6 cm×20 cm), then eluted with a linear gradient of 0.1–0.8 mM NaCl in 10 mM phosphate buffer (pH 7.0) with a flow rate of 0.5 ml/min, and absorbance at 280 nm was monitored. The fractions were collected and the activity of each fraction was measured separately. The active fractions were combined and dialyzed against 10 mM PBS (pH 7.0) for 24 h and stored at −20 ºC.

Determination of protein concentration

Protein concentration was determined by the Folin-phenol method, using bovine serum albumin as a standard protein [15].

Determination of alginate lyase activity

Mix 0.8 ml of 200 mM phosphate buffer with 0.1 ml of 10 g/L sodium alginate, then 0.1 ml enzyme solution was added. The mixture was kept at room temperature for 30 min, and the absorbance of the reaction mixture was measured at 235 nm. One unit of the enzyme activity is defined as the increase of 0.01 in absorbance at 235 nm per minute.

SDS-PAGE of alginate lyase

The molecular mass of alginate lyase was determined by SDS-PAGE [16].

Effects of temperature on enzyme activity

Alginate lyase was incubated in a water bath at a series temperature of 0, 15, 25, 30, 35, 40, 45 and 50 ºC for 1 h, respectively, then quickly cooled to 0 ºC. The enzyme activity was evaluated again to determine its optimal working temperature.

Effects of pH on enzyme activity

Three kinds of pH buffer solutions, 200 mM Na₂HPO₄-citric acid (pH 2.2–8.0), Na₂HPO₄-NaHPO₄ (pH 6.0–8.0) and 50 mM Glycine-NaOH (pH 8.6–10.6) were selected, and all experiment conditions were set at 25 ºC for 2 h. The enzyme activity was determined by incubating it in a water bath at 25 ºC for 2 h.

The determination of the enzyme kinetics curve

The enzyme, different substrates and buffer were mixed at a proportion of 0.2:0.2:1.6 (V/V/V), cultured at 25 ºC for 30 min, and the absorbance was measured at 235 nm. The concentrations of the substrates (alginate) were set to 1, 2.5, 5, 7.5, 10, 15, 20 and 30 g/L, respectively. The kinetics curves were drawn by Lineweaver-Burk double-reciprocal plot method, and the Kₘ was determined.

Effects of EDTA and metal ions on enzyme activity

Alginate (10 g/L) was dissolved in PBS buffer (pH 7.0), which contains EDTA and a different kind of metal ions (Zn²⁺, Mg²⁺, Ba²⁺ and Ca²⁺), and the enzyme was added to the above solution and incubated at 25 ºC for 1 h. The absorbance at 235 nm with or without metal ions in above solution was measured and compared.

Determination of the distribution of the molecular mass of the alginate oligosaccharide

The distribution of the molecular mass of the alginate-derived oligosaccharides was determined by PAGE method with 5% stacking gel and 22% resolving gel at 200 V for 2.5 h [17].

Substrate specificity of the alginate lyase

Five microliters of 2 g/L (M)ₙ or (G)ₙ solution was mixed with 1 ml purified alginate lyase and reacted at 25 ºC for a different time. The absorbances of the solution at 0.3, 0.6, 1, 2, 4, 6, 12 and 18 h were measured.
Results

Screening of the zymogenic strain

After zymogenic incubation, the bacteria which could degrade alginate were selected. Under the transmission electron microscope, the length of the flagellum was 3.5 times longer than that of the vibrios. The 16S rDNA sequence and the physiological and biochemical characteristics analysis indicated the stain belong to \textit{Vibrio sp.} (data not shown).

Isolation and purification of the alginate lyase

Crude enzyme precipitated from 65\% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} was separated by DEAE-Sephacel column. The enzyme activity of each fraction was tested by the ultraviolet absorption method. The first peak, which had the highest enzyme activity with 0.4 mM NaCl was collected and combined. The data are listed in Table 1. The specific activity of the pure enzyme is 5.25 times than that of the crude enzyme, and 332 times of that of the zymogenic solution.

SDS-PAGE of the pure enzyme

The alginate lyase purified from (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} precipitation and DEAE-Sephacel column was shown as a single band (Fig. 1), and the molecular mass was approximately 62.5 kDa.

Effects of temperature on enzyme activity

The enzyme activity was measured at different temperatures, and the data show that the optimal temperature was 25 °C [Fig. 2(A)]. The thermal stability results show that the enzyme was stable between 0 and 30 °C [Fig. 2(B)], but its activity decreased quickly when the temperature was higher than 30 °C, and lost 90\% activity when the temperature was higher than 50 °C.

Effects of pH on the enzyme activity

The enzyme activity was determined at different pH values, and the highest enzyme activity was observed in

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Recovery (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture fluid</td>
<td>1418.1</td>
<td>234.3</td>
<td>0.17</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} precipitation</td>
<td>87.7</td>
<td>943.8</td>
<td>10.80</td>
<td>40.3</td>
<td>36.3</td>
</tr>
<tr>
<td>DEAE-Sephacel</td>
<td>15.1</td>
<td>852.7</td>
<td>56.40</td>
<td>36.4</td>
<td>332.1</td>
</tr>
</tbody>
</table>

Fig. 1 SDS-PAGE analysis of alginate lyase purified from \textit{Vibrio sp.} YWA

1, protein standards (Da); 2, purified alginate lyase.

Fig. 2 Optimal temperature and thermal stability of the alginate lyase

(A) The optimal temperature of the enzyme was 25 °C. (B) Thermal stability of the enzyme.
phosphate buffer at pH 6.8–7.2. The enzyme activity decreased at pH>7.5 [Fig. 3(A)], and the enzyme activity was relative stable at pH 6.0–7.5 [Fig. 3(B)].

![Fig. 3](image)

**Fig. 3** Effects of different pH on alginate lyase
(A) The optimal pH of the enzyme was approximately 7.0. (B) pH stability of the enzyme.

Enzyme kinetic curve

Different concentrations of alginate were used as specific substrates to determine the enzyme kinetic properties (Fig. 4). The kinetic curve was drawn by the Lineweaver-Burk double-reciprocal plot method, and the $K_m$ was calculated as 72.73 g/L, which indicates the alginate lyase has a high activity for alginate hydrolysis.

**Effects of EDTA and metal ions on the enzyme activity**

The effects of EDTA and metal ions on enzyme activity are shown in Fig. 5. The enzyme activity of alginate was obviously increased in the presence of EDTA, Zn$^{2+}$, Mg$^{2+}$ and Ca$^{2+}$, but obviously decreased in the presence of Ba$^{2+}$.

**Substrate specificity of the enzyme**

The distribution of the molecular mass of the alginate-derived oligosaccharide was measured at different time intervals by PAGE. The results (Fig. 6) show that as time

![Fig. 4](image)

**Fig. 4** The kinetic properties of the alginate lyase

![Fig. 5](image)

**Fig. 5** Effects of metals and on enzyme activity

*P<0.05 vs. Vibrio sp. YWA only by independent sample t test using SPSS software.

![Fig. 6](image)

**Fig. 6** Electrophoresis analysis of alginate-derived oligosaccharides detected by silver staining

1, heparin oligos standard; 2–7, the hydrolysis time of 0, 1, 4, 6, 12 and 18 h, respectively.
goes on, more and more bands appeared, indicating that the content of the oligosaccharide was increasing. As the low polymerized oligosaccharide could not be stained with Alcian blue, the silver staining method was used to improve the sensitivity.

Degradation of the alginate lyase on different substrates

The specificity of alginate lyase produced by Vibrio sp. YWA was evaluated with alginate, (M)_n and (G)_n, and the data are shown in Fig. 7. The results indicated the alginate lyase has specific hydrolysis effect on (M)_n but not (G)_n in alginate.

Based on the characterization study of the alginate lyase from Vibrio sp. YWA, this alginate lyase is significantly different from others (such as alginate lases from abalone, Haliotis discus hannai, bacterium Sphingomonas sp.) in molecular weight and optimal pH and temperature [18–20]. Further study on the DNA sequence will provide new information on the structure-activity relationship study of alginate lyase. The new alginate lyase from Vibrio sp. YWA will be useful for the production of alginate-derived oligosaccharides and also as a tool for the fine structure analysis of alginate [22].

Discussion

The alginate lyase was purified to homogeneity by ammonium sulfate precipitation. Thirty-five percent of the ammonium sulfate was used to eliminate the non-active protein, and 65% of the ammonium sulfate precipitate contained crude alginate lyase. The crude enzyme was eluted using DEAE-Sephacel column by a linear gradient of 0.1–0.8 mM NaCl in phosphate buffer and a single peak with high alginate lyase activity was obtained. The purified enzyme displayed a single band in SDS-PAGE and its molecular mass was approximately 62.5 kDa.

The study on the substrate specificity has shown that the alginate lyase had a higher hydrolysis activity to (M)_n than that to (G)_n, and this result was confirmed by carbohydrate electrophoresis analysis in Fig. 7. So the enzyme was ascribed to mannuronate lyase.

References

1 Ji MH. Seaweed Chemistry. Beijing: Science Press 1997
14 Liu B, Wang CY, Zhang HR. Preparation and identification of series of

http://www.abbs.info; www.blackwellpublishing.com/abbs
15 Li JW, Xiao NG, Yu RY. Principles and Methods of Biochemistry Experiments. Beijing: Beijing House of Peking University 1979

Edited by
Iain ANDERSON