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Article

Purification and characterization of an alginate lyase from marine bacterium *Microbulbifer* sp. KIT–19

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Alginate-degrading bacteria were isolated from seawater and a-systematic classification was performed using 16S rDNA sequence. The isolated strain was identified as *Microbulbifer* sp. strain KIT-19. The properties of alginate lyase (ALG19) derived from the strain KIT-19 were investigated. ALG19 was purified by various column chromatography techniques, with specific activity of 10.1 units/mg and 7.08-fold purity. When the substrate was sodium alginate, the optimum pH of the purified ALG19 was 8.5 and optimum temperature was 40°C. The activity of the purified ALG19 increased by 2.34-fold in the presence of 100 mM Na^+. Alginate is composed of β-D-mannuronic acid (M) and 1,4-linked α-L-guluronic acid (G) residues. The activity toward poly- M blocks and poly-G blocks of purified ALG19 on the basis of relative activity scale were 123% and 91% respectively, when the decomposed activity to sodium alginate was 100%. Since ALG19 was poly-M blocks specific alginate lyase, it was thought that ALG19 belongs to the polysaccharide lyase (PL)-7 family. ALG19 could be considered to be an endo-type enzyme because it lowers the kinematic viscosity of sodium alginate from 23.5 to 1.65 cSt (mm^2/s).

Key words: Alginate lyase, Purification, Characterization, *Microbulbifer*, poly-M blocks specificity

Abbreviations: DEAE, diethylaminoethyl; DNS, 3,5-dinitrosalicylic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LBG, locust bean gum; CMC, carboxymethylcellulose; poly-M, polymannuronate; poly-G, polyguluronate; PL, polysaccharide lyase; EDTA, ethylendiaminetetraacetic acid; cSt, centi stokes.

Introduction

Seaweeds, especially the brown algae, are attracting attention as an abundant biomass next to cellulose. The most abundant carbohydrate in brown algae is alginate, which accounts for 17-45% of the total biomass[1]. Alginates are linear polysaccharides consisting of 1,4-linked α-L-guluronic acid (G) residues and its C5 epimer, β-

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D-mannuronic acid (M) residues. This polysaccharides can form homopolymeric blocks of G residues (G-blocks) or M residues (M-blocks) or heteropolymers of alternating M and G (MG - blocks)[2]. Alginate lyases are alginate-degrading enzymes, which catalyze the depolymerization of alginate[3].

In the Carbohydrate-Active enZYmes (CAZy) database, alginate lyases are classified into seven polysaccharide lyase (PL) families including families PL- 5, 6, 7, 14, 15, 17 and 18 (http://www. cazy. org/). In the CAZy database presents a classification of these enzymes in families and subfamilies based on amino acid sequence similarities, intended to reflect their structural features. Alginate lyases are also enzymatically classified into two groups based on their substrate specificity. The first group is the enzyme (EC 4.2.2.3) degrading poly-M-blocks, while the second group is the enzyme (EC 4.2.2.11) degrading poly-G-blocks. Most of the family of PL-7 alginate lyases specifically degrade poly-M-blocks and poly-G-blocks, but some enzymes degrade G and GM-blocks and M and GM-blocks[4],[5]. In addition, the same bacterium produces several alginate lyases (PL CAZy, http://www. cazy. org/). This is considered to efficiently decompose the alginic acid polymer which is normally difficult to decompose. In this study we have isolated several marine bacteria strains from seawater at the seashore of Kanazawa Port in Japan. One isolate was closely related to marine bacterium *Microbulbifer salipaludis* based on 16S ribosomal DNA. This report presents the isolation and characterization of a purified alginate lyase (ALG19) from genus *Microbulbifer*.

Materials and Methods

Isolation of marine bacterium and identification of strain KIT-19 A marine bacterium as a decomposing bacterium of *L. japonica* was isolated from the seashore of Kanazawa Port in Japan. The seawater was passed through a membrane filter (pore size, 0.45 μm, Merck, Darmstadt, Germany.) and the filter paper was soaked in a seawater-based medium (pH 8.0) containing 0.2% (w/v) $NaNO_3$, 0.001% (w/v) iron citrate *n*-hydrate, 0.006% (w/v) KH_2PO_4, and 2% (w/v) alginic acid. Single colony isolation was carried out on the same medium containing 2% (w/v) agar. The microorganisms with the highest alginate lyase activity were selected. The isolated strain named KIT-19 was grown aerobically at 30°C for 4 days. The 16S rDNA gene of strain KIT-19 was amplified by PCR from genomic DNA and sequenced using BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, USA). The obtained 16S rDNA gene sequence of strain KIT-19 was aligned automatically with closely related sequences retrieved from Aporon DB-BA 5.0 (Techno Suruga Lab., Shizuoka, Japan), GenBank, DDBJ and EMBL[6]. GenBank is a part of the International of Nucleotide Sequence Database Collaboration, which is also comprised of DDBJ and EMBL (http://www.insdc.org/). A phylogenetic tree was constructed using Neighbor-joining method and a Kimura-2-parameter model in Aporon 2.0 (Techno Suruga Lab.).

Enzyme purification The dialyzed culture broth was applied to a Toyopearl DEAE-650M column (2.5 × 20 cm; Tosoh, Japan) equilibrated with 20 mM Tris-HCl buffer (pH 9.0). After washing with the same buffer, elution of proteins was performed with linear gradient of 0–0.5 M NaCl (400 mL) in 20 mM Tris-HCl buffer (pH 9.0). The fractions containing ALG19 activity were pooled and dialyzed solution was applied to a Toyopearl DEAE-650M column (2.5 × 30 cm) equilibrated with 20 mM Tris-HCl buffer (pH 9.0) containing 0.1 M NaCl. The proteins were eluted with a 400

mL linear gradient of 0.1–0.3 M NaCl in the same buffer[6]. The fractions containing ALG19 activity were pooled. All enzyme purification steps were conducted at 4°C.

Enzyme assays The ALG19 activity was determined at 40°C for 15 min in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5% (w/v) sodium alginate as substrate. After terminating the reaction, the reducing sugar produced was determined by the 3,5-dinitrosalicylic acid (DNS) method[7] with glucose as standard. One unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar as glucose per minute under the assay conditions.

Characterization of alginate lyase To determine the pH profile of alginate lyase activity, reactions were performed at 40°C for 15 min in 0.1 M sodium citrate-NaOH buffer (pH 3.0–5.0), sodium acetate buffer (pH 5.0–6.0), potassium phosphate buffer (pH 6.0–8.0), Tris-HCl buffer (pH 8.0–9.0), and glycine-NaOH buffer (pH 9.0–11.0) containing 0.1 M NaCl.The effect of temperature on ALG19 activity was determined using 0.1 M Tris-HCl buffer (pH 8.0) at 10, 20, 30, 40, 50, 60, 70, and 80°C. Reactions were initiated by adding enzyme and progressed for 15 min[6].

To determine the viscosity reduction of the 2% (w/v) sodium alginate by the purified ALG19 (2.3 μg), the enzyme reaction was performed at 40°C for 90 min in 0.1 M Tris-HCl buffer (pH 8.0). Viscosity reduction of the 2% (w/v) sodium alginate by treatment of the ALG19 was measured using Ubbelohde SU-0001 viscometer (SIBATA, Saitama, Japan)[8].

Substrate specificity The hydrolytic activities towards various polysaccharides, sodium alginate, glucomannan, locust bean gum (LBG), carboxymethylcellulose (CMC), low-melting-point agarose, pectate, carrageenan, and laminaran were examined. Carbohydrates (0.2% (w/v)) were incubated with the purified enzyme at 40°C for 20 min in 0.1 M Tris-HCl buffer (pH 8.0) solutions, and reducing sugar concentration was determined using the DNS method. To measure the substrates specificities of the purified enzyme, M-blocks and G-blocks were prepared from hydrolysis of 5% (w/v) sodium alginate in 1 M oxalic acid at 98°C for 10 h. The insoluble material was separated into two fractions by precipitation at pH 2.85. The soluble fraction at pH 2.85 was used as the substrate of poly-M and the insoluble fraction was used as poly-G [9]. Statistical analysis with the use of Tukey test was used for significant differences of degradative activities for sodium alginate, M-blocks and G-blocks.

Effect of various cations and EDTA The purified enzyme was incubated with various cations (each at 1 mM: Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, Na^{+} , and K^{+}) at 30°C for 30 min, and the effect of EDTA on alginate lyase activity was examined using EDTA at a final concentration of 5 mM. The remaining activity in the samples was determined by measuring the activities of ALG19 under standard conditions in the presence of various additional reagents. The activity obtained using the reaction measurement without any additional reagent was recorded as 100%. Furthermore the enzyme activity was determined with enzyme reaction mixtures containing various concentrations of NaCl (0 – 400 mM). Alginate lyase activity was measured after incubating the enzyme mixtures at 40°C for 15 min in 0.1 M Tris-HCl buffer (pH 8.0)

Thin-layer chromatography (TLC) A reaction mixture composed of 0.2% (w/v) sodium alginate, 0.1M Tris-HCl buffer (pH 8.0), and 0.26 units of enzyme was incubated at 40°C for 30min. After the

reaction, hydrolysis products from sodium alginate was separated on 0.2 mm Silica Gel 60 (Merck, Darmstadt, Germany.) aluminum plates with n-butanol/acetic acid/water (1:1:1, v/v) as eluent. Sugars were detected by spraying the TLC plates with 5% (v/v) sulfuric acid in ethanol, followed by heating at 200°C until color developed.

Results and Discussion

Description of Microbulbifer sp. KIT-19

The almost complete 16S rDNA sequence (1,461 bp) of the strain KIT-19 was analyzed. The phylogenic tree constructed from the sequence data showed that the strain KIT-19 appeared within the evolutionary radiation encompassing genus *Microbulbifer* (Fig. 1). The homologies between the KIT-19 16S rDNA sequence and the 16S rDNA sequences of strains *Microbulbifer elongatus*[10] and *Microbulbifer salipaludis*[11] were 99.5% and 98.3%, respectively. The phylogenetic tree suggested that the strain KIT-19 belonged to the *Microbulbifer* genus. Therefore, it was named as *Microbulbifer* sp. KIT-19.

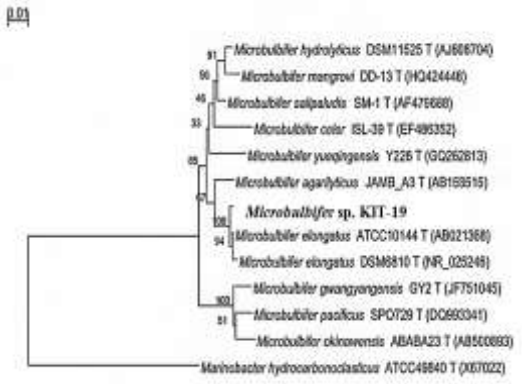

Fig. 1. Neighbor-Joining tree based on 16S rDNA sequences of *Microbulbifer* sp. KIT-19 and other bacteria. The unrooted tree was constructed by Neighbor Joining analysis. Numbers at nodes indicate levels of bootstrap support (%). Scale bar: 0.01 substitutions per nucleotide position.

Purification of alginate lyase

The ALG19 was purified to homogeneity from cultures of *Microbulbifer* sp. KIT-19 and the subsequent two-step column chromatographic procedure. The molecular mass of the purified enzyme was estimated to be approximately 29.2 kDa using SDS-PAGE analysis against molecular mass standards (Fig. 2). Approximately 7.08 fold purification to a specific activity of 10.1 U/mg protein was obtained for the ALG19 activity when measured at pH 8.0 at 40°C by the DNS method. Viscosity reduction of the 2% (w/v) sodium alginate using ALG19 was measured by Ubbelohde SU viscometer. As a result, the kinematic viscosity of the enzyme reaction mixture was 1.65 cSt (mm^2/s). However, the kinematic viscosity of the reaction mixture without enzyme was 23.5 cSt (mm^2/s). These numerical values represent the time required for the liquid to move a certain distance, for example, 1.65 cSt (mm^2/s) represents a rapid decrease in sodium alginate viscosity. Endo-type enzymes have been reported in various polysaccharide-degrading enzymes, including alginate lyase, to cause a decrease in substrate viscosity[12, 13, 14, 15]. This experimental result suggested the possibility that ALG19 is an endo-type enzyme.

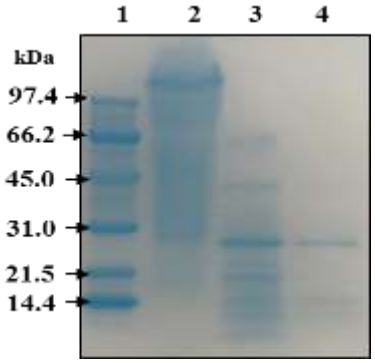

Fig. 2. SDS-PAGE of purified ALG19. The protein was stained with Coomassie brilliant blue (lane 1-4). Lane 1, protein mass markers (in kDa); lane 2, culture broth; lane 3, partial purified enzyme eluted from first DEAE 650M column; lane 4, purified enzyme eluted from second DEAE 650M column.

Effect of pH on the activity and stability of ALG19

The alginate lyase activity was apparent over a narrow range of pH values, with more than 90% of the maximum activity detected between pH 8.0 and 9.0 (Fig. 3A). This pH profile was similar to AlgMsp from *Microbulbifer* sp. 6532A[16]. The purified ALG19 was a semi-alkaline enzyme, with an optimum pH of 8.5.

To examine the stability of the ALG19 at different pH values, the enzyme was preincubated at 30°C for 30 min in various buffers (50 mM) and the residual activities were assayed. The enzyme was stable, with more than 80% of the original activity detected throughout the wide pH range from 5.0 to 10 (data not shown).

Effect of temperature on the activity and stability of ALG19

The optimum temperature of ALG19 for the reaction with sodium alginate as substrate was detected at around 40°C and pH 8.0 in 0.1 M Tris-HCl buffer (Fig. 3B). The thermal stability of the purified ALG19 was assayed in 0.1 M Tris-HCl buffer (pH 8.0) after heating for 30 min at various temperatures. The enzyme was stable up to 30°C, and above this temperature the residual activity decreased gradually. More than 60% of the original activity remained even after heating at 40°C. In general, the optimum temperature of the enzymes from various marine bacteria, *Vibrio* sp. JAM-A9m[17], *Sphingomonas* sp. ZH0[18], and *Saccharophagus* sp. Myt-1[19], are around 30°C to 40°C. The present enzyme had an optimum temperature at 40°C, similar to other enzymes.

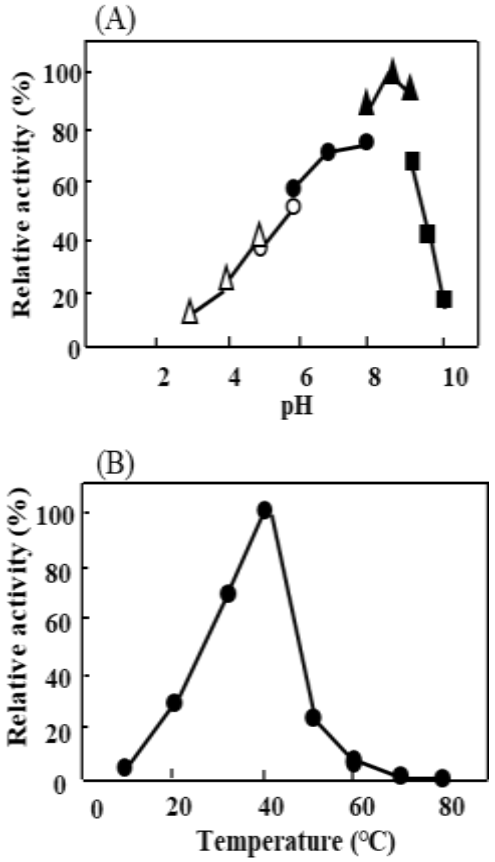

Fig. 3. Effects of (A) pH and (B) temperature on the purified ALG19 activity. The activity was measured in 0.1 M sodium citrate-NaOH buffer (pH 3.0–5.0, *open triangles*), 0.1 M sodium acetate buffer (pH 5.0–6.0, *open circles*), 0.1 M potassium phosphate buffer (pH 6.0–8.0, *closed circles*), 0.1 M Tris-HCl buffer (pH 8.0–9.0, *closed triangles*), and 0.1 M glycine-NaOH buffer (pH 9.0–11, *closed squares*) at 40°C for 40 min. The relative activity was calculated assuming the activity at a pH 8.5 (*closed triangles*) as 100%. The effect of temperature on ALG19 activity was determined by incubating the reaction mixtures at 10, 20, 30, 40, 50, 60, 70, and 80°C in 0.1 M Tris-HCl buffer (pH 8.0). Reactions were initiated by adding ALG19 (32 ng/μL)

and terminated after 15 min. The relative activity was calculated assuming the activity at 40°C as 100%.

Effect of metal ions and EDTA

Purified ALG19 was incubated with various cations (each at 1 mM) at 30°C for 30 min, and residual activity was then assayed by the DNS method. The various metal ions, K^+, Mg^{2+}, and Mn^{2+} had a slight inhibitory effect. In addition, ALG19 was inhibited by Fe^{2+}, losing 92% activity. Ca^{2+}, Ni^{2+} and Zn^{2+} could inhibit the enzyme activity between 20 to 30% as shown in Table 1. Among the investigated metal ions, none had activating effects on the activity of ALG19. However, when the relative activity was calculated by assuming that the activity in 0 mM NaCl was 100 %, the alginate lyase activities at various NaCl concentrations of 25 mM, 50 mM, 100 mM, 200 mM, and 400 mM were 175, 188, 234, 213, and 190%, respectively. The purified ALG19 was a salt-activated enzyme, similar to several alginate lyases previously reported[5, 16, 20]. The effect of 10 mM EDTA on the enzyme activity of ALG19 was negligible. Based on our results, for ALG19, monovalent metal ion such as Na^+ was needed for its optimal activity but divalent metal ions such as Ca^{2+} and Mg^{2+} were not essential.

Table 2. Effects of metal ions and EDTA on the activity of the purified ALG19

Reagents (1 mM)	Remaining activity (%)
Control	100
Na^+	101
Na^+ (100 mM)	234
K^+	98.5
Ca^{2+}	71.4
Mg^{2+}	94.5
Mn^{2+}	96.7
Fe^{2+}	8.31
Ni^{2+}	81.0
Zn^{2+}	75.7
EDTA (10 mM)	99.2

S***ubstrate specificity***

The enzyme exhibited highest activity on sodium alginate, although glucomannan, LBG, CMC, low-melting-point agarose, pectate, carrageenan, and laminaran were not hydrolyzed[6]. In addition, we used poly M- and poly-G-blocks, which were prepared following the method of Haug *et al*.[9], and sodium alginate as substrates. The purified ALG19 exhibited the highest activity toward poly- M-blocks (123%) on the basis of relative activity scale, when the decomposed activity to sodium alginate was 100%. The AGL19 activity on the poly G-blocks (91%) was less than that of sodium alginate (100%) (Fig. 4). This poly M-block specific enzyme activity of ALG19 was similar to the alginate lyase belonging to family PL-7 from *Microbulbifer* sp. 6532A[16], *Vibrio* sp. JAM-A9m[17], *Vibrio* sp. O2[21] and *Pseudomonas aeruginosa*[22]. Furthermore, these alginate lyases containing AlgMsp from *Microbulbifer* sp. 6532A were activated in Na^+. On the other hand,alginate lyases from *Microbulbifer* sp. Q7specifically degrade poly-G-blocks[23], and AlgL17 from *Microbulbifer* sp. ALW1 is a poly-M-specific oligoalginate lyase [24]. AlgL17 from *Microbulbifer* sp. ALW1 was classified into the family PL-17. Alginate lyases from genus *Microbulbifer* are classified in families PL-7, 14 and 17. The family members of PL-14 and PL-17 are mainly exo-oligoalginate lyases and various types of oligoalginate lyases, respectively.Since the amino acid sequence of ALG19 is unknown, the family cannot be classified properly. However, because ALG19 is a poly-M-block-specific alginate lyase, and poly-M-block-specific alginate lyases are currently classified only in family PL-7 (PL CAZy, http://www. cazy. org/), it is possible that ALG19 belongs to family PL-7

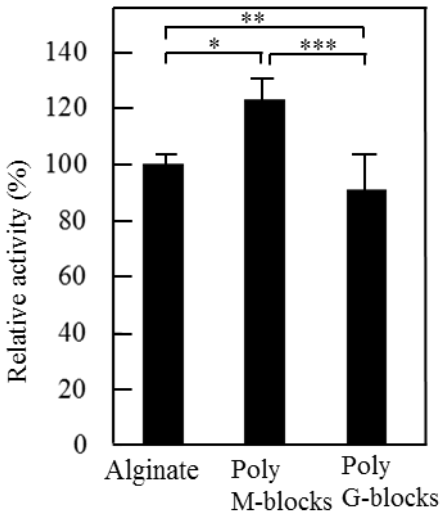

Fig. 4. Substrate specificity of the purified ALG19 for alginate, poly-M-blocks and poly-G-blocks. Various substrates were incubated with the purified enzyme at 40°C for 20 min in 0.1 M Tris-HCl buffer (pH 8.0) solutions, and reducing sugar concentration was determined using the DNS method. The error bars represent the average of three independent experiments. The error bars are means ±S.D.
Tukey tests, *P<0.05, **P>0.05, ***P<0.05

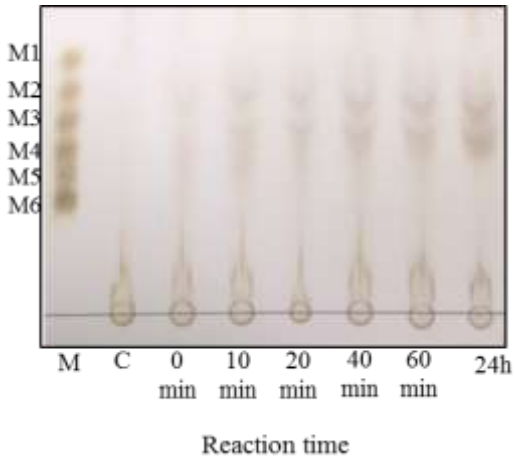

Fig. 5. TLC analysis of degradation products of sodium alginate with the purified ALG19. The numbers on the horizontal axes on the graphs indicate the reaction time. Lane M, standard oligosaccharides; M1, mannose; M2, mannobiose; M3, mannotriose; M4, mannotetraose; M5, mannopentaose; M6, mannohexaose. Lane C, control without enzyme.

Analysis of hydrolysis products by TLC

For digestion of 0.2% (w/v) sodium alginate, a 1.0 mL reaction mixture in 0.1 M Tris-HCl buffer (pH 8.0) and 0.34 units of enzyme was incubated at 40°C for 0–60 min. Hydrolysis products released from sodium alginate by 0.34 units of purified ALG19 contained oligoalginate, based on TLC analysis. The degree of polymerization (DP) 2-4 of oligoalginate was equivalent to the degradation products from sodium alginate at each timepoint (Fig.5). This result revealed that ALG19 alginate lyase-mediated degradation of alginate produced DP 2-4 of oligoalginate as the most abundant product. This characteristic of ALG19 is considered to be a property suitable for recovering DP 2-4 of oligoalginate.

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