Purification and Characterization of Extracellular Alginate Lyase from Enterobacter cloacae M-1

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An alginate lyase from the culture supernatant of Enterobacter cloacae M-1 was purified by ammonium sulfate precipitation, cation-exchange chromatography (SP-Toyopearl), and gel filtration (Ultrigel Aca44). The final preparation thus obtained showed a single band on SDS–PAGE. The purified enzyme had the molecular weight of 38,000 and 32,000 by SDS–PAGE and gel filtration, respectively. The pI of the enzyme was 8.9. The optimum pH and temperature for the enzyme reaction were around 7.8 and 30°C, respectively. The enzyme was unstable on heating. EDTA completely inhibited the enzyme activity, but the activity was completely restored by the treatment with CaCl₂. The enzyme was specific for poly-guluronate and produced several kinds of unsaturated oligomers from the guluronate. This suggested that the enzyme could be classified as an endo poly-guluronate lyase.

Alginates are extracted from brown seaweeds. They are acidic polysaccharides composed of (1→4)-linked β-D-mannuronic acid and (1→4)-linked α-L-guluronic acid residues, arranged in three types of block structures, poly-guluronate (poly-G), poly-mannuronate (poly-M), and heteropolymeric random sequences (poly-MG).1–3 Alginates are widely used for biotechnological, medical, food-industrial, and other uses. Although alginate solutions have high viscosity, the enzymatic and acidic degradations of alginate solution have low viscosity like water. The resultant degradation products have novel uses. It has been reported that enzymatically degraded algines were found to promote bifidobacterial growth,4 and the germination and shoot elongation of some plants.5–7

Alginate-degrading enzymes have been isolated from many sources including marine algae, marine molluscs, and microorganisms.6 All these enzymes, except only one,3 are lysases, catalyzing β-elimination that cleaves the glycosidic bond and leads to formation of an unsaturated uronic acid residue. Alginate lyases have a preference for either L-guluronic acid or D-mannuronic acid residues and are called guluronate lyase or mannuronate lyase, respectively.

Although many alginate lyases have been reported, the definition of enzyme specificity has not been fully understood.6 To understand this issue, we now report, as a first step, the purification and characterization of the extracellular endo poly-guluronate lyase obtained from Enterobacter cloacae (E. cloacae) M-1.

Materials and Methods
Substrates. Sodium alginate (Duck Algin) produced by Kibun Food Chemifa Co., Ltd. was used in this experiment. The mannuronic acid/guluronic acid (M/G ratio) of the alginate was 0.94. The ratio was measured by the method of Haug et al.8 Poly-mannuronate (poly-M) and poly-guluronate (poly-G) were prepared from the sodium alginate after partial acid hydrolysis.9 These were then dialyzed against deionized water using SpectraPor dialysis membrane (MWCO: 500) and lyophilized. Circular dichroism analysis9 established that the poly-M contained 91% M, and the poly-G contained 81% G.

Preparation of the crude enzyme. E. cloacae M-1 was isolated from soil, and the culture supernatant was used as the enzyme source for purification. The method of isolation of E. cloacae M-1 and enzyme production will be reported elsewhere.

Enzyme assay and measurement of protein concentration. For the enzyme assay, 0.5 ml of 1% sodium alginate solution, adjusted to pH 7.8 with 50 mM Tris–HCl buffer containing 1 mM CaCl₂, was poured into a test tube and incubated at 30°C for 5 min. The reaction was started by the addition of 0.5 ml of enzyme solution. After incubation for 30 min, the reaction mixture was immediately heated at 100°C for 10 min to stop the reaction, and 0.2 ml of the mixture was withdrawn to another test tube. The amount of unsaturated material produced by the lyase action was measured by the TBA reaction.

One unit of enzyme activity was defined as the amount of enzyme required to liberate the equivalent of 1 μmol of β-formylpyruvic acid per 1 min; 0.01 μmol of β-formylpyruvic acid produces an A₅₄₆ of 0.290 in the TBA reaction.

The protein concentration in the purification process of enzyme was measured by the absorbance at 280 nm, assuming that the absorbance at 280 nm at the concentration of 1 mg of protein/ml is 1.0.

Thin-layer chromatography (TLC). To characterize the reaction products, after the enzyme reaction mixture was treated with cation-exchange resin (Amberlite IR-200C), the sugar sample was put on a TLC plate of silica gel 60 (Merck). The TLC plate was then developed with a solvent system of 1-butanol-formic acid–water (4:6:1, v/v) by the ascending method. The spots of the uronic acids were stained by heating the TLC plate at 150°C for 5 min after spraying with conc. sulfuric acid. The unsaturated sugars were also stained by TBA reagent.10

Results
Taxonomic characterization of strain M-1
Figure 1 is a micrograph of strain M-1. Table 1 also describes the morphological and biochemical characteristics.

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1 To whom offprints should be requested.
Abbreviations: G, guluronate; M, mannuronate, TBA, thiobarbituric acid; CBB, Comassie brilliant blue; E. cloacae, Enterobacter cloacae; β-ME, β-mercaptoethanol.
Table I. Morphological and Biochemical Characteristics of Strain M-1

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<td>Decarboxylation of lysine</td>
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of the strain. The strain M-1 was identified as Enterobacter cloacae (E. cloacae) M-1 at the Japan Food Research Laboratories.

Purification of extracellular alginate lyase

All procedures were done at 4°C. Purification steps of extracellular alginate lyase from E. cloacae M-1 are summarized in Table II.

1) (NH₄)₂SO₄ precipitation. On the first step in purification of the enzyme from the culture supernatant, 7 liters of the supernatant solution was brought to 90% saturation by adding (NH₄)₂SO₄ and left overnight. The precipitate was collected by centrifugation, dissolved in 150 ml of 10 mm Tris–HCl buffer (pH 7.8) containing 1 mm CaCl₂, and dialyzed against 10 mm Tris–HCl buffer (pH 7.8). Insoluble materials were removed by centrifugation.

ii) SP-Toyopearl 650M column chromatography. The dialyzed enzyme obtained above was put on a column (16 × 180 mm) of SP-Toyopearl 650M, equilibrated with 10 mm Tris–HCl buffer (pH 7.5), at the flow rate of 40 ml/h. After the column was washed enough with the same buffer, the absorbed proteins, as shown in Fig. 2, were eluted with a linear gradient of 0 to 0.8 m NaCl in the same buffer (total volume, 300 ml). The eluate was fractionated into 5 ml portions. The active fraction (tube numbers, 40–48) was dialyzed against deionized water and lyophilized.

iii) Ultrogel AcA 44 column chromatography. After the enzyme powder was dissolved in 1 ml of 10 mm Tris–HCl buffer (pH 7.8) containing 1 mm CaCl₂, the solution was put on a Ultrogel AcA 44 column (15 × 880 mm), equilibrated with 10 mm Tris–HCl buffer (pH 7.8) containing 0.1 m NaCl and 1 mm CaCl₂, at the flow rate of 15 ml/h. The eluate was fractionated into 5 ml portions. The active fraction (tube numbers, 29–31) was dialyzed against deionized water and lyophilized. The enzyme powder was dissolved in 20 ml of 50 mm Tris–HCl buffer (pH 7.8) containing 1 mm CaCl₂.

Starting from the culture supernatant, a 21,000-fold increase in specific activity was achieved with a yield of 25%. The purified enzyme gave a single band on SDS–PAGE (Fig. 3).

Measurements of molecular weight, isoelectric point, and amino-terminal sequence

The molecular weight of the enzyme was 38,000 by SDS–PAGE in the presence or absence of β-mercaptoethanol (β-ME) (Fig. 3). Furthermore, the non-denatured molecular weight, as found from gel filtration through TSK gel G3000SW, was 32,000 (Fig. 4).
The purified enzyme was incubated with SDS (Purified Enzyme) or 1% β-mercaptoethanol (+ β ME) at 100°C. SDS-PAGE was done on an 12% polyacrylamide gel by the method of Laemmli, and the protein was stained with Coomassie brilliant blue (CBB) R-250. Standard, molecular weight standards from 14,300 to 57,200 (BDH).

The pI of the enzyme was 8.9 by CBB R-250 staining (Fig. 5A) and activity staining (Fig. 5B).

Approximate 100 pmol of the enzyme was used for SDS-PAGE, and the protein was blotted onto Immobilon Transfer Membranes (Millipore Co.). After blotting, the membrane was stained with CBB R-250 to detect the protein band. The area containing the enzyme was cut out and put on an automated protein sequencer/PTH analyzer system (Model 477A) from Applied Biosystems. The amino-ter-

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\text{Fig. 5. Isoelectric Focusing of Alginase Lyase.}
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Isoelectric focusing was done using a Multiphor II system (Pharmacia) and Ampholine PAG plates (pH 3.5-9.5, Pharmacia). Protein was stained with CBB R-250 (A), and the enzyme activity on gel was detected by a substrate-containing agarose-gel overlay assay using ruthenium red (B). An isoelectric focusing calibration kit (pH 3-10) (Pharmacia) was used as standard proteins for pI measurement.

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\text{Fig. 6. Enzymatic Properties of Alginase Lyase.}
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A: Effects of pH on the enzyme activity. The alginate lyase activity was measured in McIlvaine buffer (pH 4.0-8.0, ■), 50 mM Tris-HCl (pH 7.0-9.0, ●), Atkins-Pantin (pH 7.5-10.5, ▲) buffers at 30°C.

B: Effects of temperature on the enzyme activity. The enzyme activity was measured at various temperatures from 4° to 60°C and pH 7.8 (50 mM Tris- HCl buffer).

C: Effects of pH on the enzyme stability. After the enzyme solution was maintained at various pHs (McIlvaine buffer, pH 4.0, ■; 50 mM Tris HCl buffer, pH 7.9, ●; Atkins-Pantin buffer, pH 8.11, ▲) and 20°C for 1 h, the remaining activity was assayed.

D: Effects of temperature on the enzyme stability. After the enzyme was treated at various temperatures (4-60°C) and pH 7.8 (50 mM Tris- HCl buffer) for 1 h, the remaining activity was assayed.
Fig. 7. Effects of Metal Compounds on EDTA-treated Enzyme.
The enzyme solution was treated with EDTA at 1 mM of final concentration at pH 7.8 and 30°C for 30 min, and then the enzyme activity was assayed. Next, each of metal compounds was added to the EDTA-treated enzyme solution to bring 2 mM of final concentration, and the mixture was kept at 30°C for 30 min. Then, the enzyme activity was assayed and expressed as the relative activity of control.

Fig. 8. TLC of Enzymatic Degradation Products from Sodium Alginate.
The crude enzyme (2 ml including 0.05 unit/ml) or the purified enzyme (the same) was added to 1% alginate solution (2 ml) adjusted to pH 7.8 with 50 mM Tris-HCl buffer containing 3 mM CaCl₂. Then, the enzyme reaction was done at 30°C for 24 h, and then the resultant products were tested by TLC. C: crude enzyme; P: purified enzyme.

Enzymatic properties

i) Effects of pH and temperature on enzyme activity.
As shown in Fig. 6A, the enzyme had maximal activity at approximately pH 7.8. The optimum temperature for the activity was around 30°C (Fig. 6B).

ii) Effects of pH and temperature on enzyme stability.
The enzyme was stable between pH 6 to 10, but showed low activity when the enzyme was maintained in 50 mM Tris-HCl buffer (Fig. 6C). The enzyme activity decreased with increasing temperature, and no enzyme activity was observed after treatment at 60°C (Fig. 6D).

Reactivation of EDTA-treated enzyme by metal compounds

Most of the bacterial alginate lyases have required some metal ions for maximal activity. To test their effect on the activity, the enzyme was treated with EDTA and metal compounds. Figure 7 shows the effects of metal compounds on the EDTA-treated enzyme. The original enzyme activity was almost completely inhibited with EDTA (EDTA-treated in Fig. 7), but the activity of EDTA-treated enzyme was restored by treatment with metal compounds, especially CaCl₂, AlCl₃, and MnCl₂.

Comparison of crude enzyme with purified enzyme on the degradation of sodium alginate

The crude and purified enzymes gave almost the same products from the alginate (Fig. 8). Therefore, this result together with the purification steps suggests that E. cloacae M-1 extracellularly produced one kind of alginate lyase.

Substrate specificity of alginate lyase

To identify the substrate specificity of the enzyme, the enzyme was incubated with either poly-G or poly-M. The enzyme predominantly attacked the poly-G rather than the poly-M (Fig. 9A). Figures 9B and 9C show the characterization of the degradation products. In the action of
the enzyme on the poly-G, the TBA-positive main product
appeared at the second spot from the top on TLC (Fig
9C). However, the TBA positive spot was undetectable
with the poly-M (Fig 9B). On the other hand, the analogous
polypeptides such as pactate were not degraded by the
enzyme (data not shown).

Discussion
We purified the extracellular alginate lyase from E.
cloacae M-1 to a single band on SDS–PAGE (Fig. 3).
The molecular weight of the enzyme was 38,000 by SDS–PAGE
in the presence or absence of β-ME (Fig. 3), and was 32,000
by gel filtration (Fig. 4), suggesting that the enzyme is probably composed of a single polypeptide. Most of the
alginate lysates are also composed of a single polypeptide
except bacterial alginate lyase except bacterial alginate lyase
which has two subunits. The pl of our enzyme was 8.9 (Fig. 5). This is the same as intracellular and extracellular poly-guluronate lysates from
Klebsiella pneumoniae (aerogenes) (pI 8.9).13 Klebsiella sp.,
as well as E. cloacae M-1, has been classified into the family
of Enterobacteriaceae. However, the molecular weight of
our enzyme was slightly different from that of the Klebsiella
intracellular enzyme, which has been reported to be 31,600
by SDS–PAGE and 28,000 by gel filtration.1,5
Almost all of alginate lysates have optimum pH around
neutral (pH 7–8), and the optimum temperatures of many
alginate lysates are 20–40°C.15,16–19 The optimum pH and
temperature of our enzyme also corresponded to these
values (Figs. 6A and 6B). These of the Klebsiella intracellular
enzyme were around 7.0 and 37°C, respectively, and these
values do not significantly deviate from these of our enzyme.
However, the Klebsiella enzyme activity had a broad pH
optimum with a sharp drop of activity below pH 6.0 and
above pH 7.5. Our enzyme activity indicated a narrower
pH optimum and higher pH optimum (around 7.8, Fig.
6A) than the Klebsiella enzyme. In addition, the activity of
Klebsiella enzyme was higher at 37°C than at 30°C, in sharp
contrast to that of our enzyme (Fig. 6B). Accordingly, our
enzyme is clearly different from the Klebsiella intracellular
enzyme.

Several alginate lysates have been cloned, and the nucleotide
sequences of the genes were identified.20–25 Interestingly,
the seven residues of the amino-terminal sequence of our
enzyme completely matched to the deduced amino acid
sequence of the Klebsiella extracellular alginate lyase
gene.25 The molecular weight of the cloned enzyme was
31,400 by SDS–PAGE,25 but was slightly smaller than that
of our enzyme. Hence, this work is the prelude to cloning
our enzyme to elucidate whether or not our enzyme is
identical with the Klebsiella extracellular enzyme.

The enzyme from E. cloacae M-1 rapidly decreased the
viscosity of alginate solution during the very beginning of
the reaction (data not shown). Moreover, the absorbance
at 548 nm (TBA reaction) on the poly-G degradation
increased with the progress of reaction (Fig. 9A). Thus, our
enzyme was characterized as an endo poly-guluronate lyase.

Our enzyme significantly acted on the poly-M (Fig. 9A).
Some studies demonstrated that endo poly-guluronate
lyases also slightly attacked poly-M.15,18,26–29 One reason
may be that the poly-M contains significant proportions of
the G. Another reason is perhaps that the Klebsiella
extracellular enzyme has cleaved not only G-G linkage but
also G-M linkage among the four possible glycosidic
linkages in alginate (G-G, M-M, G-M, and M-G).30 From
this standpoint, our enzyme was also likely to attack the
G-M linkage but not the M-M linkage in the poly-M used,
since the poly-M contained 9% of the G. Hence, these
studies prompted us to investigate the exact substrate
specificity of our enzyme. To this end, we first attempted
to confirm that the enzyme could cleave at least the G-G
linkage but not the M-M linkage. To prepare either more
enriched M content or more enriched G content substrates,
we tried to use the method of Takahashi et al., which can
get complete homogeneity of both poly-M and poly-G.31
But, we could not obtain the same result for some unknown
reasons.

The major end-product of the endo-acting alginate lysases
appear to be the unsaturated triuronic.1,26–29,32 The main
product of our enzyme action on the poly-G was obviously
the second spot from the top on TLC (Fig. 9C). This spot
was corresponded to the migration of the unsaturated triuronic
standard, indicating that the main end-product was unsaturated
triuronic. On the other hand, the end-products (Fig. 9C, lane S)
of enzyme action between on the intact alginate differed from that (Fig. 9C, lane 24) on the poly-G. That is to say, the end-products from the intact alginate in comparison with the poly-G revealed large amounts
of unsaturated tetra- to heptauronides. Since these unsaturated
uronides are unable to be digested with our enzyme, the
structural analysis of these uronides can elucidate the
substrate specificity of the enzyme.

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