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Purification and Characterization of Endo Poly (α-L-Guluronate) Lyase in the Enzyme System from *Flavobacterium multivolum*

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An alginate lyase was purified from a crude enzyme of *Flavobacterium multivolum* K-11 by successive column chromatographies, such as cation exchange, chromatofocusing, and gel filtration. The enzyme thus obtained migrated as a single band on SDS-PAGE. The relative molecular mass of the enzyme was 43-kDa by SDS-PAGE and 41-kDa by HPLC gel filtration chromatography. The isoelectric point of the enzyme was 8.7. The enzyme exhibited maximum activity at pH 8.0 and 40°C, and was stable in the pH range of 6.0 to 9.0 and at temperatures up to 30°C. The enzyme activity was remarkably inhibited by chemical compounds such as EDTA, PCMB, MIA, TNBS, and *N*-bromosuccinimide. The enzyme was specific for poly-guluronate and produced several kinds of oligomers. Thus, the results suggested that the enzyme was classified as endo poly (α-L-guluronate) lyase (EC 4.2.2.11).

Keywords: alginate lyase, guluronate lyase, alginic acid, *Flavobacterium multivolum*

Alginates, extracted from brown seaweeds, are a kind of hetero poly-uronide which is composed of (1→4) linked α-L-guluronic acid and (1→4) linked β-D-mannuronic acid residues. The residues are at least arranged in block structures which are homo-polymeric poly-guluronate (G-block), homo-polymeric poly-mannuronate (M-block), and hetero-polymeric random sequence (MG-block). The alginate and its derivatives have been extensively used for the cosmetic, pharmaceutical and food industries (for review, see Gacesa, 1992; Murata *et al.*, 1993).

Alginic acid is difficult to apply for novel utilization such as a beverage, because an aqueous solution of the alginate has a high viscosity even at a concentration of 0.5%. To this end, we have previously reported, as a first step, a method for depolymerization of alginate using a crude enzyme system of *Flavobacterium multivolum* K-11 (*F. multivolum*) (Takeuchi *et al.*, 1994). The enzyme system rapidly decreased the viscosity of the alginate solution at the beginning of the reaction and produced several kinds of oligosaccharides at the final stage of reaction. However, the characterization of the enzyme from *F. multivolum* is not fully understood. In addition, there are few studies on the characterization of the purified enzyme, although many sources, such as marine algae, marine mollusuca, and microorganisms, are known to produce alginate-degrading enzymes (Gacesa, 1992; Sutherland, 1995). For these reasons, we purified an alginate lyase, which is specific to poly-guluronate, i.e., poly (α-L-guluronate) lyase (EC 4.2.2.11), from the crude enzyme system of *F. multivolum* and investigated some properties of the purified enzyme.

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Materials and Methods

Substrates and materials A crude powder of alginate-degrading enzyme from *F. multivolum* K-11 was purchased from Nagase Biochemicals, Ltd., Fukuchiyama, Kyoto. Alginate, "Duck Algin 350M-T" produced by Kibun Food Chemifa Co., Ltd., Tokyo was used in this experiment. The mannuronic acid/guluronic acid (M/G ratio) of this alginate was 0.94, obtained as described (Haug *et al.*, 1974). Three blocks, namely the G-block, M-block and MG-block, were prepared from the alginate as described (Haug *et al.*, 1967). The guluronic acid and mannuronic acid contents of the three were determined by circular dichroism analysis using a JASCO J-720 spectropolarimeter (Morris *et al.*, 1980). The result established that the G-block contained 89% guluronic acid, the M-block contained 92% mannuronic acid, and the MG-block contained 57% mannuronic acid. D-Mannuronic acid lactone was purchased from Siguma Chemicals Co., St. Louis, Mo, CM-Toyopearl 650M was obtained from Tosoh, Tokyo. Polybuffer Exchanger 94 and Polybuffer 96 were from Pharmacia, Uppsala, Sweden. Ultrogel AcA-54 was from LKB, Bromma, Sweden.

Enzyme assay The alginate-degrading enzyme activity was measured by thiobarbituric acid (TBA) reaction (Preiss & Ashwell, 1962). The assay was performed essentially as described previously (Nibu *et al.*, 1995) except that the enzyme reaction was carried out at 37°C. Briefly, 1% sodium alginate dissolved in 50 mM Tris-HCl buffer (pH 8.0) was mixed with an equal volume of enzyme solution. This mixed solution was incubated at 37°C for 30 min. Next, 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 A_{548} of 0.290.

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

Purification of enzyme All the procedures were carried out at about 4°C.

Step 1: preparation of crude enzyme solution Twelve grams of crude enzyme powder from *F. multivolum* K-11 was dissolved in 600 ml of 1 mM phosphate buffer (pH 6.3) and dialyzed against the same buffer.

Step 2: CM-Toyopearl 650M column chromatography The dialyzed enzyme solution was centrifuged at 6200×*g*, and the supernatant (total activity, 1850 units) was applied to a CM-Toyopearl 650M column (2.6×40 cm) equilibrated with 1 mM phosphate buffer (pH 6.3). After the column was washed with about 600 ml of the same buffer, the enzyme was eluted from the column with a linear gradient from 0 to 0.5 M of NaCl in the same buffer (500 ml each) at a flow rate of 120 ml/h. The eluate was fractionated into 10-ml portions. The enzyme having alginate-degrading activity separated into two peaks, namely tube numbers 19 to 25 (total activity, 838 units) and tube numbers 39 to 46 (total activity, 454 units). Tube numbers 19 to 25 were combined, concentrated by ultrafiltration using a YM-3 membrane (Amicon, Bevery, Mass.) and was lyophilized.

Step 3: first chromatofocusing The lyophilized enzyme was dissolved in 10-fold diluted Polybuffer 96 (pH 7.0) and then loaded on the Polybuffer Exchanger 94 column (1.3×30 cm) equilibrated with ethanolamine-HCl buffer (pH 9.6). The enzyme was eluted with 10-fold diluted Polybuffer 96 (pH 7.0) at a flow rate of 30 ml/h. The eluate was fractionated into 5-ml portions. The active fractions, tube numbers 15 to 18 (total activity, 596 units), were combined, dialyzed against deionized water, and lyophilized.

Step 4: second chromatofocusing The lyophilized enzyme was dissolved in 10-fold diluted Polybuffer 96 (pH 7.0) and applied to chromatofocusing. The alginate-degrading enzyme was eluted with 10-fold diluted Polybuffer 96 (pH 7.0) at a flow rate of 30 ml/h. The eluate was fractionated into 5-ml portions. The active fractions, tube numbers 21 to 23 (total activity, 570 units), were combined, dialyzed against deionized water, and lyophilized.

Step 5: Ultrogel AcA-54 column chromatography In order to remove Polybuffer 94 in the enzyme solution, the lyophilized enzyme was dissolved in 3 ml of 40 mM phosphate buffer (pH 6.7) containing 0.24 M NaCl and then applied to a column (1.6×85 cm) of Ultrogel AcA-54 equilibrated with the same buffer. The elution was done at a flow rate of 15 ml/h. The eluate was fractionated into 3-ml portions. The fractions of alginate-degrading enzyme, tube numbers 33 to 38 (total activity, 319 units) were combined.

Estimation of relative molecular mass and isoelectric point (pI) The relative molecular mass of the enzyme was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Weber & Osborn, 1969) and also estimated by gel filtration chromatography as described previously (Nibu *et al.*, 1995) except that the gel filtration used two columns of a Protein Pak 300 (7×300 mm, Waters Division of Millipore). The pI of the purified enzyme was measured as described previously (Nibu *et al.*, 1995).

Amino acid analysis The purified enzyme was hydrolyzed *in vacuo* with 6 N HCl at 110°C for 24 h. The resultant amino acids were determined with a Hitachi amino acid analyzer type 835-50.

Thin-layer chromatography (TLC) TLC was performed as described previously (Takeuchi *et al.*, 1994; Nibu *et al.*, 1995).

Results and Discussion

Purification of enzyme The results of the purification procedures are summarized in Table 1. For purification of alginate lyase from *F. multivolum* K-11, the crude enzyme was subjected to sequential CM-Totopearl, chromatofocusing (twice), and Ultrogel AcA-54 column chromatographies. The purified enzyme revealed a single band on SDS-PAGE (Fig. 1). Consequently, the alginate lyase was purified 16.4-fold with a yield of 17.2% on the basis of the crude enzyme solution. However, the specific activity of the purified enzyme fell from 25.4 to 17.6 after Ultrogel AcA-54 column chromatography. As the cause, it is thought that the enzyme was partially inactivated because of its instability against pH and temperature as described below.

Relative molecular mass and pI The relative molecular mass of the enzyme was estimated to be 41-kDa by HPLC

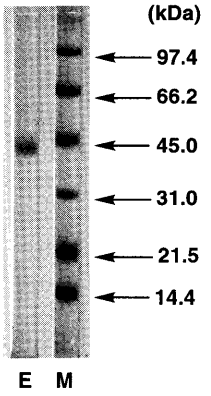

Fig. 1. Analysis of the purified enzyme by SDS-PAGE and Coomassie brilliant blue R-250 staining. Lane E, purified enzyme; lane M, relative molecular mass (M_r) markers.

Table 1. Summary of the purification of alginate-degrading enzyme from *Flavobacterium multivolum* K-11.

Purification step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification (-fold)
Crude enzyme solution	1730	1850	1.07	100	1.0
CM-Toyopearl 650M	85.5	838	9.8	45.3	9.2
First chromatofocusing	34.2	596	17.4	32.2	16.3
Second chromatofocusing	22.4	570	25.4	30.8	23.7
Ultrogel AcA-54	18.1	319	17.6	17.2	16.4

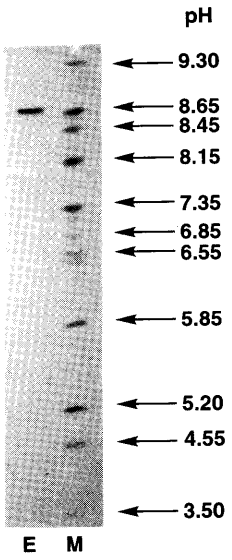

Fig. 2. Estimation of pI of purified enzyme. Lane M, pI standards; lane E, purified enzyme.

gel filtration analysis (data not shown) and 43-kDa by SDS-PAGE (Fig. 1), indicating that the enzyme was a single peptide. The relative molecular mass of the enzyme was similar to that of a guluronate lyase from *Vibrio* (Takeshita *et al.*, 1993, 1995). This value was larger than those of guluronate lyases from *Enterobacter cloacae* M-1 (Nibu *et al.*, 1995), *Klebsiella aerogenes* (Lange *et al.*, 1989), and an unidentified bacterium (i.e., the A1-III lyase) (Hisano *et al.*, 1993, 1994; Murata *et al.*, 1993) but was smaller than that from *Vibrio harveyi* AL-128 (Kitamikado *et al.*, 1992). On the other hand, the pI of our enzyme was estimated to be 8.7 (Fig. 2). The pI of the enzyme was very close to those of the guluronate lyases from *Enterobacter cloacae* M-1 (Nibu *et al.*, 1995) and *Klebsiella aerogenes* (Caswell *et al.*, 1986) but not from that of *Vibrio harveyi* AL-128 (Kitamikado *et al.*, 1992).

General properties of the purified enzyme The effects of pH and temperature on purified enzyme activity are shown in Fig. 3A and B, respectively. The optimum pH for the activity was around 8.0 (Fig. 3A), and the optimum temperature for the activity was around 40°C (Fig. 3B). The optimum pH of our enzyme was similar to those of three guluronate lyases (Kitamikado *et al.*, 1992; Hisano *et al.*, 1993; Nibu *et al.*, 1995) but was slightly different from that of *Vibrio* (Takeshita *et al.*, 1993, 1995). The optimum temperature of our enzyme was similar to that from *Klebsiella aerogenes* (Lange *et al.*, 1989) but not to those of two guluronate lyases (Takeshita *et al.*, 1993; Nibu *et al.*, 1995). We have previously reported that the optimum pH of the crude enzyme of *F. multivolum* was around 7 (Takeuchi *et al.*, 1994), raising the possibility that the enzyme system from *F. multivolum* may contain several alginate lyases. On the other hand, the effects of pH and temperature on the stability of the purified enzyme are shown in Fig. 3C and D, respectively. The enzyme was stable between pH 6.0 and pH 9.0 (Fig. 3C). The enzyme was stable up to 30°C, but no enzyme activity was obtained after the treatment at 50°C for 2 h (Fig. 3D). This enzyme was more thermostable than the guluronate lyase from *Enterobacter cloacae* M-1 (Nibu *et al.*, 1995) but was more unstable than

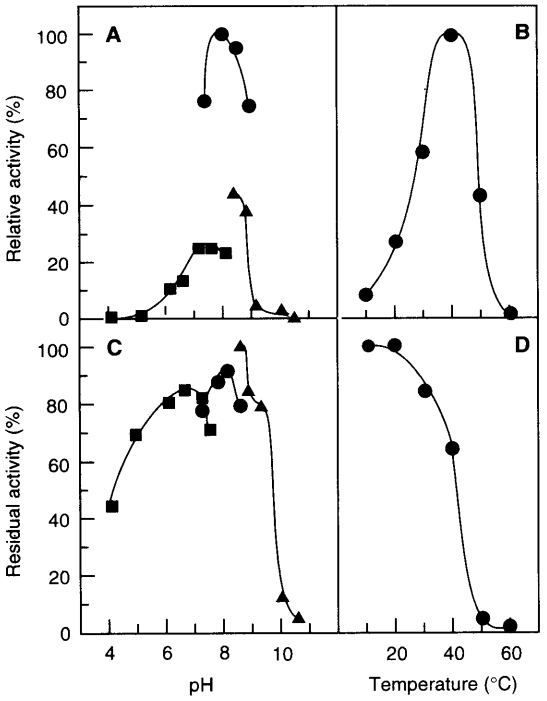

Fig. 3. Enzymatic properties of the purified enzyme. A: Effect of pH on the enzyme activity. The activity was determined at various pH values, using McIlvaine buffer (■), Tris-HCl buffer (●), and Atkins-Pantin buffer (▲), at 37°C for 30 min. B: Effect of temperature on the enzyme activity. The enzyme activitiy was determined at various temperatures at pH 8.0 in Tris-HCl buffer. C: Effect of pH on the enzyme stability. Enzyme solutions were incubated at various pH values, using McIlvaine buffer (■), Tris-HCl buffer (●), and Atkins-Pantin buffer (▲), at 25°C for 2 h, and the residual activity was assayed at pH 8.0 in Tris-HCl buffer. D: Effect of temperature on the enzyme stability. The enzyme was incubated at various temperatures at pH 8.0 in Tris-HCl buffer for 1 h, and the residual activity was determined.

Table 2. Effects of chemical compounds on enzyme.

Compound	Residual activity (%)
None	100
EDTA	13
SDS	122
PCMB	33
MIA	6
N-Ethylmaleimide	99
2-Mercaptoethanol	109
TNBS	31
N-Bromosuccinimide	0

After the enzyme solution containing a test compound (1×10^{-3} M) was preincubated in Tris-HCl buffer at pH 8.0 and 25°C for 15 min, the residual activity was assayed. Residual activities are expressed as percentage of the activity in the absence of the test compound. EDTA, ethylenediamineteraacetic acid; SDS, sodium dodecyl sulfate; PCMB, *p*-chloromercuribenzoic acid; MIA, monoiodoacetic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

the marine bacterial guluronate lyase (Takeshita *et al.*, 1993).

In addition, the purified enzyme contained amino acids in the order of Gly (15.2 mol%)>Asx (12.4 mol%)≒Ser (12.2 mol%)>Thr (10.2 mol%), and other amino acids were less than 10 mol% (data not shown).

Effects of chemical compounds Table 2 shows the effects of chemical compounds on the purified enzyme. The enzyme activity was decreased by the addition of EDTA, *p*-chloromercuribenzoic acid (PCMB), monoiodoacetic acid

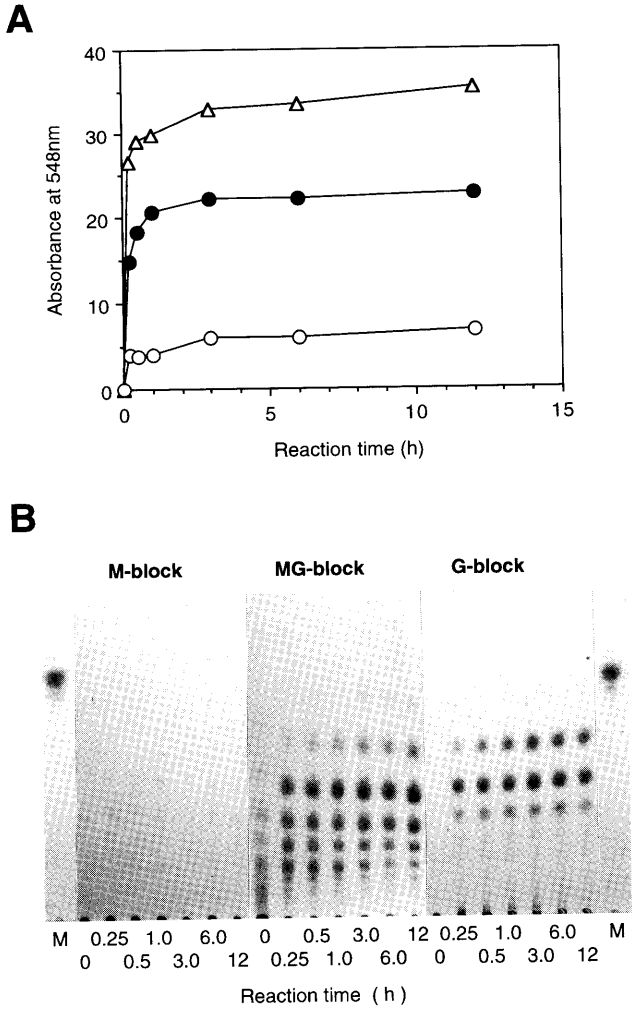

Fig. 4. Time-course of the enzyme reaction toward G-, MG- and M-blocks. A : The course of enzyme reaction followed by TBA reaction. △, G-blocks; ●, MG-blocks; ○, M-blocks. B: The course of enzyme reaction followed by TLC staining with sulfuric acid. Lane M, D-mannuronic acid lactone.

(MIA), 2,4,6-trinitrobenzenesulfonic acid (TNBS), and *N*-bromosuccinimide, while treatment with SDS, *N*-ethylmaleimide, and 2-mercaptoethanol was found to have no significant effect on the enzyme activity. This result suggests that amino acid residues containing the SH-group and triptophan residue of the enzyme participated in the activity.

Substrate specificity of the purified enzyme To examine the specificity of the enzyme, 100 μl of enzyme solution containing 2.1 units was added to each 1.0 ml of 1% G-block (G content; 89%), MG-block (M content; 57%), and M-block (M content; 92%), and the reaction was then continued at pH 8.0 and 37°C. At certain intervals of time (0, 0.25, 0.5, 1, 3, 6, and 12 h), 100 μl of the reaction mixture was taken out and immediately heated at 100°C for 5 min to inactivate the enzyme. The unsaturated uronic acids produced by the enzyme reaction were assayed by the TBA reaction (Fig. 4A). The increase in the absorbance at 548 nm was rapid during the beginning of the reaction but gradually levelled off thereafter, although the purified enzyme degraded the three blocks in the order of G-block>MG-block≫M-block. Subsequently, to detect the enzymatic degradation products, we performed TLC with the reaction mixture (Fig. 4B). Several kinds of oligomers were explicitly observed from the G- and MG-blocks with significant difference but not from the M-block. The main products were five or six kinds of oligomers from the MG-block (MG-block in Fig. 4B), whereas there were three kinds of oligomers from the G-block (G-block in Fig. 4B). These findings, together with our previous results (Takeuchi *et al.*, 1994), suggested that this enzyme was classified as an endo poly (α-L-guluronate) lyase (EC 4.2.2.11).

Conclusion In the present study, we purified an alginate-degrading enzyme from *F. multivolum* K-11 to electrophoretic homogeneity and revealed some properties of the enzyme. The purified enzyme was classified as an endo poly (α-L-guluronate) lyase (EC 4.2.2.11) based on its substrate specificity. However, we suspect that the crude enzyme may contain another alginate lyase. This possibility was supported by the purification steps and by the difference in the optimum pH between the crude enzyme and the purified enzyme. To use the alginate-oligomer produced by the action of the crude enzyme for industrial applications, we are studying the structural analysis of the oligomers, and also the purification and characterization of another enzyme from *F. multivolum* K-11.

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