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Characterization of a Novel Alginate Lyase from *Flavobacterium multivolum* K-11

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An alginate lyase was purified from an extracellular enzyme (commercial preparation) of *Flavobacterium multivolum* K-11 by successive column chromatographies, such as cation exchange, chromatofocusing, and gel filtration. The purified enzyme migrated as a single band on SDS-PAGE and analytical isoelectric focusing. The molecular weight of the enzyme was 32,000 by SDS-PAGE and 33,000 by HPLC gel filtration chromatography, and the pI of the enzyme was 8.2 on isoelectric focusing. The enzyme exhibited maximum activity at pH 7.5 and 40°C, and was stable between pH 6.0 and 9.0, and at temperatures up to 20°C. The enzyme activity was remarkably inhibited by chemical compounds such as SDS, MIA, TNBS, and *N*-bromosuccinimide, while EDTA and PCMB had no effect on the enzyme activity. The enzyme decomposed both the G-block (guluronic acid content; 89%) and the M-block (mannuronic content; 92%) at nearly equal rates, and produced several kinds of unsaturated oligomers. Because such activity of alginate lyase has not been reported, we believe that this is a novel alginate lyase.

Keywords: alginate lyase, alginate, *Flavobacterium multivolum*

Alginates, extracted from brown seaweeds, are a kind of hetero poly-uronide composed of (1→4) linked α-L-guluronic acid and (1→4) linked β-D-mannuronic acid residues. The residues are arranged in block structures which are either homo-polymeric poly-guluronate (G-block) or homo-polymeric poly-mannuronate (M-block) or hetero-polymeric random sequence (MG-block). The alginate and its derivatives have been extensively used for biotechnological, medical, food-industrial, and other uses (Gacesa, 1992; Murata *et al.*, 1993). It has been demonstrated that the alginates depolymerized by alginate-degrading enzymes were then found to promote bifidobacterial growth (Akiyama *et al.*, 1992), germination and shoot elongation of some plants (Natsume *et al.*, 1994; Tomoda *et al.*, 1994; Yonemoto *et al.*, 1993).

Alginate-degrading enzymes have been isolated from many sources including marine algae, marine molluscs, and microorganisms (Gacesa, 1992; Sutherland, 1995). Two types of alginate lyase have been reported. The alginate lyases preferentially cleave either poly-mannuronate or poly-guluronate. Accordingly, they are classified as poly(β-D-mannuronate) lyase (EC 4.2.2.3) or poly(α-L-guluronate) lyase (EC 4.2.2.11), respectively.

We have previously reported a method for the depolymerization of alginate using a crude alginate lyase (enzyme system) from *Flavobacterium multivolum* K-11 (*F. multivolum*) (Takeuchi *et al.*, 1994). The enzyme system degraded alginate into several kinds of unsaturated oligosaccharides consisting of mannuronic acid, guluronic acid and both. Thus, we presumed that the enzyme system consisted of more than one alginate lyase. One of them was endo poly(α-L-guluronate) lyase (Ochi *et al.*, 1995; Takeuchi *et al.*, 1997). However, the characteristics of others are not yet known. In the present study, we have fractionated the enzyme system of *F. multivolum* and report the characteristics of a novel alginate lyase that degrades both the G-block and M-block substrates at nearly equal rates.

Materials and Methods

Enzyme An enzyme powder, an extracellular enzyme originating from *F. multivolum* K-11, was purchased from Nagase Biochemicals, Ltd. (Osaka).

Substrates Alginate, "Duck Algin" produced by Kibun Food Chemifa Co., Ltd. (Tokyo), was used in this experiment. The mannuronic acid/guluronic acid (M/G) ratio of the alginate was 0.94 which was determined according to the method of 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 blocks were determined by circular dichroism analysis using a JASCO J-720 spectropolarimeter (Tokyo) (Morris *et al.*, 1980). The result indicated that the G-block contained 89% guluronic acid, the M-block contained 92% mannuronic acid, and the MG-block contained 57% mannuronic acid.

Assay for alginate lyase activity The assay was essentially performed as described previously (Ochi *et al.*, 1995). The amount of unsaturated saccharides by lyase action was measured by thiobarbituric acid (TBA) reaction (Preiss & Ashwell, 1962). One unit of the enzyme activity was defined as the enzyme amount of forming 1 μmol of β-formylpyruvate per min. Ten nano moles of β-formylpyruvate give an A_{548} of 0.29 in the TBA assay (Preiss & Ashwell, 1962).

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Abbreviations: G, α-L-guluronic acid; M, β-D-mannuronic acid; TBA, thiobarbituric acid; SDS, sodium dodecyl sulfate; MIA, monoiodoacetic acid; TNBS, trinitrobenzene sulfonic acid; EDTA, ethylenediaminetetraacetic acid; PCMB, *p*-chloromercuribenzoic acid.

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

Purification of alginate lyase All the procedures were carried out at 4°C.

Preparation of crude enzyme solution A 12 g sample of enzyme powder from *F. multivolum* K-11 was dissolved in 600 ml of 1 mM phosphate buffer, pH 6.3 (buffer A), and dialyzed against buffer A overnight.

CM-TOYOPEARL 650M column chromatography The dialyzed enzyme solution was centrifuged at 6200×*g*, and the supernatant was applied to a CM-TOYOPEARL 650M (Tosoh, Tokyo) column (2.6×40 cm) equilibrated with buffer A. After the column was washed with about 600 ml of buffer A, the enzyme in the column was eluted with a linear gradient of NaCl from 0 to 0.5 M in the same buffer (total volume; 1000 ml), at a flow rate of 120 ml/h. The eluate was fractionated into 10-ml aliquots. The fractions containing alginate lyase were pooled, concentrated by ultrafiltration using a YM-3 membrane (Amicon, Bevery, Mass.), and lyophilized.

Chromatofocusing The lyophilized enzyme was dissolved in 10-fold diluted Polybuffer 96 (pH 7.0, Pharmacia, Uppsala, Sweden) and then subjected to a Polybuffer Exchanger 94 (Pharmacia) 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 aliquots. The active fractions were combined, dialyzed against deionized water, and lyophilized.

Ultrogel AcA-54 column chromatography In order to remove the Polybuffer 96 from the enzyme solution, the lyophilized enzyme was dissolved in 3 ml of 40 mM phosphate buffer (pH 6.7) containing 0.24 M NaCl (buffer B), and then applied to a column (1.6×85 cm) of Ultrogel AcA-54 (LKB, Bromma, Sweden) that was pre-equilibrated with buffer B. The elution was done at a flow rate of 15 ml/h. The eluate was fractionated into 3-ml aliquots. The fractions of alginate lyase were pooled.

Estimation of molecular weight The molecular weight of the enzyme was estimated under a denaturing condition by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Weber & Osborn, 1969). Molecular weight markers (low range, Bio-Rad Laboratories, Hercules, Calif.) were used as standard proteins. The gels were stained with Coomassie brilliant blue R-250.

The molecular weight of the enzyme under a non-denaturing condition was estimated by gel filtration. The gel filtration chromatography was performed using the method with two series-linked Protein Pak 300 columns (7×300 mm, Waters Co., Milford, Mass.) using a HPLC (TOSOH CCPM with UV-8000 TOSOH UV detector). Phosphate buffer (100 mM, pH 6.8) containing 0.1 M Na_2SO_4 was used as an elution buffer. A molecular weight marker kit (Oriental Yeast Co., Tokyo) was used as a standard marker for proteins.

Estimation of pI The isoelectric point (pI) of the purified enzyme was measured by analytical isoelectric focusing (IEF) using a Multiphor II system (Pharmacia) and Ampholine PAG plates (pH 3.5–9.5, Pharmacia) as recommended in the manufacturer's instructions. The gels were stained with Coomassie brilliant blue R-250.

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

Substrate specificity of alginate lyase To examine the substrate specificity of the enzyme, 0.1 ml of the purified enzyme solution (2.2 units) was added to each 1.0 ml of a 1% solution of the G-block, the MG-block and the M-block preparations. The enzyme reaction was then performed at pH 8 and 37°C. At defined intervals of time (0, 0.25, 0.5, 1, 3, 6 and 12 h), 0.1 ml 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 determined by the TBA reaction. On the other hand, the reaction mixture was subjected to thin-layer chromatography (TLC) for the characterization of the reaction products. The TLC was performed as described previously (Takeuchi *et al.*, 1994; Nibu *et al.*, 1995).

Results and Discussion

Purification of alginate lyase The enzyme solution (total activity, 1850 units) dialyzed against buffer A was applied to a CM-TOYOPEARL 650M column. Most of the alginate lyase was adsorbed on the ion exchanger and was eluted by a linear gradient of NaCl. The alginate lyase activity was separated into two peaks, namely fraction numbers 19 to 25 (total activity, 838 units) containing endo poly(α-L-guluronate) lyase (Ochi *et al.*, 1995; Takeuchi *et al.*, 1997) and fraction numbers 39 to 46 (total activity, 454 units). For further purification, the latter fractions were pooled, concentrated, and lyophilized. The lyophilized powder was dissolved in 10-fold diluted Polybuffer 96 (pH 7.0), and the solution was applied to a chromatofocusing column with Polybuffer Exchanger 94. The alginate lyase separated into three peaks. The major peak contained 224 units of the enzyme along with the two minor peaks of 13 and 57 units of the enzyme, respectively. The major active fraction, i.e., tube numbers 29 to 32 (total activity, 224 units), were pooled, dialyzed against deionized water, and lyophilized. The final purification was carried out by gel filtration with an Ultrogel AcA-54 column to exchange the buffer. The alginate lyase was obtained in a single peak. This alginate lyase preparation was purified 29.6-fold relative to the crude enzyme solution, and the specific activity of the enzyme was 31.7 units/mg (Table 1). The purified enzyme could be resolved as a single

Table 1. Summary of the purification of EndoMGase[a] 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	28.9	454	15.7	24.5	14.7
Chromatofocusing	6.94	224	32.3	12.1	30.2
Ultrogel AcA 54	4.98	158	31.7	8.5	29.6

[a]EndoMGase, endo-poly(β-D-1,4-mannuronide-α-L-1,4-guluronide) lyase.

band on both SDS-PAGE (Fig. 1) and IEF (Fig. 2). In addition, we, at this time, suggest endo-poly(β-D-1,4-mannuronide-α-L-1,4-guluronide) lyase (EndoMGase) as a temporary name for the purified enzyme, because the enzyme degrades both the M-block and G-block equally as described below.

Molecular weight and pI of EndoMGase The molecular weight of EndoMGase was estimated by both SDS-PAGE and gel filtration on a Protein Pak 300 column. The SDS-PAGE determination showed a molecular weight of 32,000 (Fig. 1). The gel filtration analysis revealed a molecular weight of 33,000 under a non-denaturing condition (Fig. 3), indicating that the enzyme exists as a monomeric peptide. The pI of EndoMGase under a non-denaturing condition was 8.2 (Fig. 2).

Amino acid composition of EndoMGase Table 2 shows the amino acid composition of EndoMGase compared with those of endo-polyguluronate lyase (EndoGase) obtained from *F. multivolum* K-11. The order of the amino acid composition was His>Asx>Glx>Lys≒Gly for EndoMGase, but Gly>Asx≒Ser>Thr>Val for EndoGase (Takeuchi *et al.*, 1997). Based on these results, it is suggested that both enzymes differed remarkably from each other in amino acid composition.

General properties of EndoMGase The effects of pH and temperature on the enzyme activity are shown in Figs. 4A

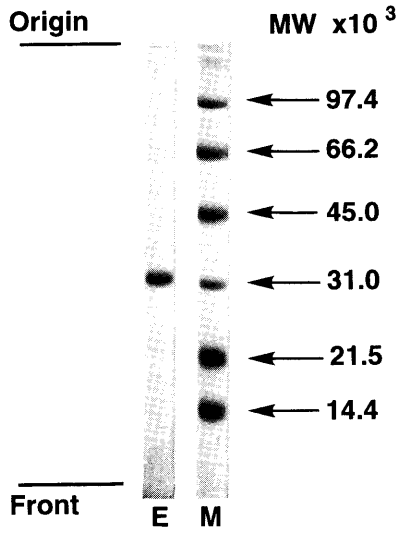

Fig. 1. SDS-PAGE of the purified EndoMGase by Coomassie brilliant blue R-250 staining. Lane E, purified EndoMGase; lane M, molecular weight markers.

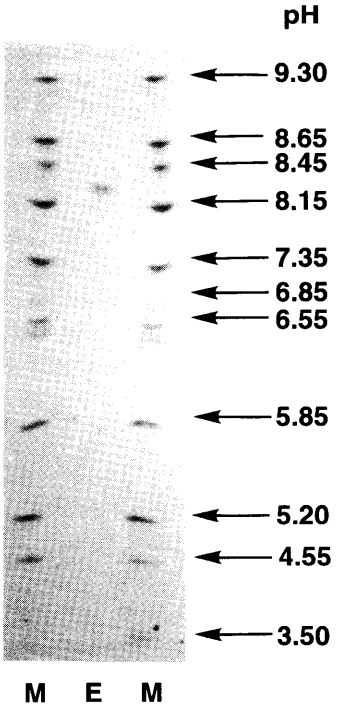

Fig. 2. Estimation of pI of EndoMGase by isoelectric focusing. Lane E, EndoMGase; lane M, pI standards (Pharmacia).

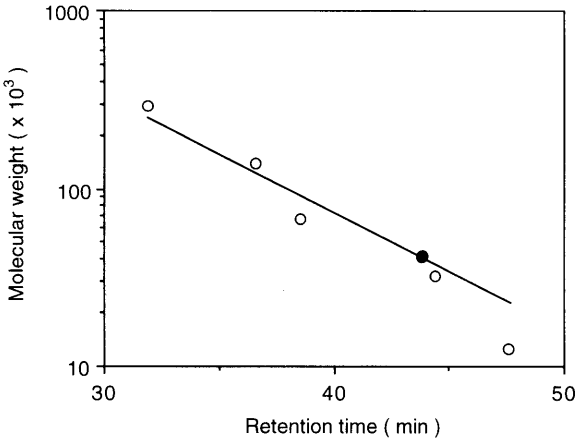

Fig. 3. Estimation of molecular weight of EndoMGase by gel filtration column chromatography. ○, molecular weight standards (Oriental Co.); ●, EndoMGase.

Table 2. Amino acid compositions of EndoMGase and EndoGase from *Flavobacterium multivolum* K-11.

Amino acids	Mol percentage (%)	
	EndoMGase[a]	EndoGase[b]
Asx	13.8	12.4
Thr	6.6	10.2
Ser	8.9	12.2
Glx	12.0	7.1
Gly	9.3	15.2
Ala	8.0	5.9
Val	4.4	8.9
Met	1.4	n.d.[c]
Ile	6.6	5.6
Leu	5.1	8.0
Tyr	4.0	0.7
Phe	5.8	3.3
Lys	9.7	6.7
His	16.6	1.0
Arg	2.6	2.9

[a]EndoMGase, endo-poly(β-D-1,4-mannuronide-α-L-1,4-guluronide) lyase. [b]EndoGase (Takeuchi *et al.* 1997), endo-poly(α-L-1,4-guluronide) lyase. [c]n.d., not determined.

and 4B, respectively. The optimum pH for enzyme activity was 7.5 (Fig. 4A), and the optimum temperature for such activity was 40°C (Fig. 4B). The effects of pH and temperature on enzyme stability are shown in Figs. 4C and 4D, respectively. The enzyme was stable between pH 6.0 and pH 9.0 (Fig. 4C). The enzyme was also stable up to 20°C; however, no enzyme activity was obtained after the treatment at 60°C for 1 h (Fig. 4D).

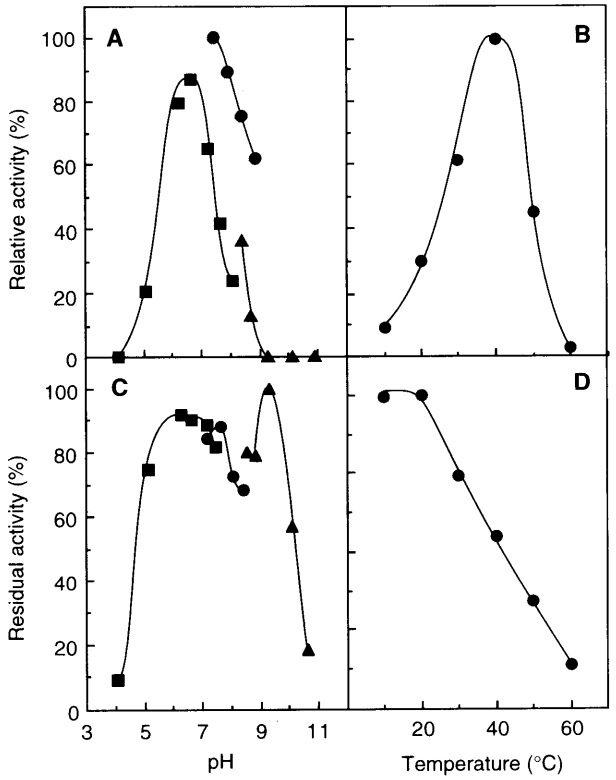

Fig. 4. Enzymatic properties of EndoMGase. A: Effect of pH on the enzyme activity. The activity was assayed at various pHs, using McIlvaine buffer (■), Tris-HCl buffer (●), and Atkins-Pantin buffer (▲), at 37°C for 30 min. B: Effect of temperature on the enzyme activity. The enzyme activity was determined at various temperatures at pH 8.0 in Tris-HCl buffer. C: Effect of pH on the enzyme stability. The enzyme solution was incubated at various pHs, 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 3. Effects of chemical compounds on EndoMGase.[a]

Compounds	Residual activity (%)
None	100
EDTA	103
SDS	3
PCMB	102
MIA	0.5
N-Ethylmaleimide	81
2-Mercaptoethanol	124
TNBS	43.5
N-Bromosuccinimide	2.5

[a] EndoMGase, endo-poly(β-D-1,4-mannuronide-α-L-1,4-guluronide) lyase. 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. The residual activities are expressed in percent in the absence of the test compound.

Effects of chemical compounds on EndoMGase Table 3 shows the effects of chemical compounds on the enzyme. The enzyme activity was decreased by the addition of SDS, MIA, TNBS and *N*-bromosuccinimide. The result suggests that this enzyme activity is attributed to the amino acid residues containing the SH-group and the tryptophan residues. On the other hand, EDTA and PCMB had no effect on the enzyme activity, although a weak effect was observed with *N*-ethylmaleimide and 2-mercaptoethanol.

Substrate specificity of EndoMGase Figure 5A shows the course of the EndoMGase reaction followed by the TBA reaction toward the M-, MG- and G-blocks. Among the three blocks, the increase in the absorbance at 548 nm was initially rapid, followed by a gradual leveling-off. Interestingly, the

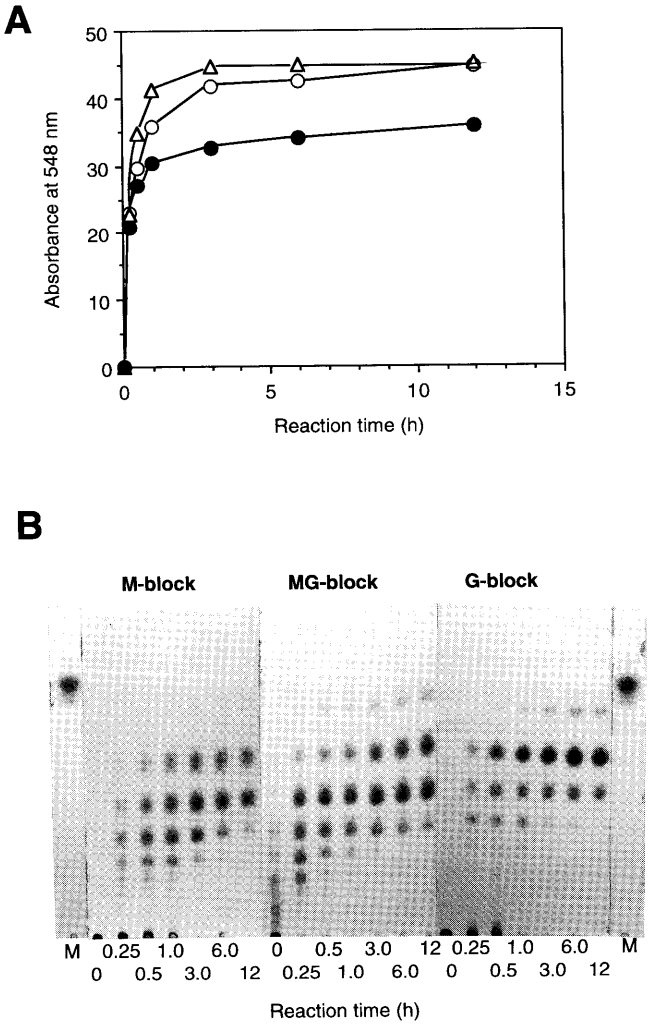

Fig. 5. Time-course of reaction of EndoMGase toward the G-block, MG-block, and M-block. A: The enzyme was incubated with the G-block, M-block, and MG-block. Reaction time is indicated in this figure. The course of the enzyme reaction was followed by TBA reaction (△, G-block; ●, MG-block; ○, M-block); B: Subsequently, the degradation products were subjected to TLC staining with sulfuric acid. Lane M, D-mannuronic acid lactone (Sigma Chemicals Co.).

enzyme reacted with both the G-block and M-block nearly equally, although the enzyme showed a slightly lower degree toward the MG-block than the above two. Figure 5B shows the course of the EndoMGase reaction followed by TLC toward the three blocks. The reaction products were slightly different between the three substrates, though a series of unsaturated oligosaccharides were produced from the three during the entire course of the reaction. These results indicate that the enzyme was an alginate lyase which acted on both the M-block and G-block. Thus, our enzyme showing such specificity is a novel alginate lyase.

Because the alginate lyases so far reported have proved to be either poly(β-D-mannuronide) lyase or poly(α-L-guluronide) lyase (Gacesa, 1992; Sutherland, 1995), we describe several possible interpretations for the substrate specificity of our enzyme.

First, it is considered to be due to the result of contamination by other alginate lyases in the purified enzyme. However, the possibility of such contamination was ruled out by both SDS-PAGE (Fig. 1) and IEF (Fig. 2) analyses, because the enzyme preparation was observed as a single band.

The second possibility is as follows. The M-block and G-block used in this study contained 8% guluronic acid and 11% mannuronic acid, respectively. However, the two substrates are suitable for a study of the substrate specificity of alginate lyase. The two purified poly(α-L-guluronide) lyases from *Enterobacter cloacae* M-1 (Nibu *et al.*, 1995) and *F. multivolum* K-11 (Takeuchi *et al.*, 1997) degraded the G-block substrate, but not the M-block substrate. On the other hand, one purified poly(β-D-mannuronide) lyase (2.1 units) from abalone acetone powder (Sigma, St. Louis, Mo.) was incubated in the 1% M-block solution under pH 8.0 and 37°C for 24 h. The enzyme degraded the M-block, but not the G-block (data in our laboratory).

The third possibility is as follows. β-D-Mannuronic acid and α-L-guluronic acid have considerably similar chemical structures. Therefore, it is reasonable that an enzyme showing broad specificity can act on both substrates.

In conclusion, we have purified and characterized a novel alginate lyase from *F. multivolum* K-11 that cannot be classified as poly(β-D-mannuronide) lyase (EC 4.2.2.3) or poly(α-L-guluronide) lyase (EC 4.2.2.11), because the enzyme degrades the M-block and G-block substrates at nearly equal rates. Thus, we designated the enzyme as endo-poly(β-D-mannuronide-α-L-guluronide) lyase.

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