Studies on Endo-Alginate Lyases and Their Applications

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ABBREVIATIONS

CD; circular dichroism

DP; degree of polymerization

Δ; 4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronic acid

EDTA; ethylenediaminetetraacetic acid

EndoGase; endo-poly(1,4-α-L-guluronide) lyase, EC 4.2.2.11

EndoMase; endo-poly(1,4-β-D-mannuronide) lyase, EC 4.2.2.3

EndoMGase; endo-poly(1,4-β-D-mannuronide-1,4-α-L-guluronide) lyase

G; L-guluronic acid

HPLC; high-pressure liquid chromatography

IEF; isoelectric focusing

K-alg; potassium alginate

kDa; kilodalton

M; D-mannuronic acid

MIA; monoiodoacetic acid

Mr; molecular mass

Na-alg; sodium alginate

NBS; *N*-bromosuccimide

NH_4-alg; ammonium alginate

PCMB; *p*-chloromercuribenzoic acid

PGA; propylene glycol ester of alginic acid

pI; isoelectric point

SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS; sodium dodecyl sulfate

TBA; thiobarbituric acid

TLC; thin-layer chromatography

TNBS; 2,4,6-trinitrobenzenesulfonic acid

GENERAL INTRODUCTION

The objective of this research is to find various kinds of alginate-degrading enzymes (alginate lyases) for expanding further the utilization of alginate, to investigate some properties of the purified enzymes for developing enzymology, and to apply the enzyme for the preparation of degradation products of alginate.

Alginate (alginic acid) is the principal ingredient of brown seaweeds (algae), and is also biosynthesized by certain microorganisms (Cohen and Jonstong, 1964; Linker and Jones, 1966; Evance and Linker, 1973;). Alginic acid is a (1→4)-linked acidic polysaccharide consisting of β-D-mannuronic acid (M) and α-L-guluronic acid (G). The polysaccharide is composed of three blocks (three types of block structures, see Fig. GI-1), namely M-block (Poly-mannuronate, M-rich region), G-block (poly-guluronate, G-rich region), and MG-block (hetero-polymeric random sequence of M and G, MG-region) (Haug *et al.*, 1967). From such the complex structure of the polysaccharide, the author expected the presence of enzymes with a variety of substrate specificities for the depolymerization of the alginate. However, the alginate lyase so far reported have proved to be either poly-mannuronide lyase (EC 4.2.2.3, see Fig. GI-2) or poly-guluronide lyase (EC 4.2.2.11), and hetero-polymeric lyase has not yet been reported.

Alginates and their propylene glycol esters, which have metal-chelating and highly viscous properties, are widely used in food and pharmaceutical industries (Preiss and Ashwell, 1962). On the other hand, acidic oligosaccharides derived from alginate were found to elicit germination-, shoot elongation-, and root growth-promoting activities (Natsume *et al.* 1994; Yonemoto *et al.*,1993; Tomoda *et al.*, 1994). The oligosaccharides also stimulated the growth of Bifidobacteria (Akiyama *et al.*, 1992). Moreover, it has been reported that M-block had potent biological effects in several biological systems

(Fujihara and Nagumo, 1993; Otteriei *et al.*, 1991). Thus, the enzymatic procedure for the degradation of alginate has been required to utilize alginate oligosaccharides in practice.

Although many sources such as marine algae and microorganisms are known to produce alginate-degrading enzymes, their enzymatic characterizations are not revealed in detail when the author started this study. Moreover, the study relating to the commercial application for the depolymerization of alginate has not been published to date. In addition, alginate lyases are also essential for the characteristics fine structures of alginates. From circumstances in the study, this research involves the following detailed objectives.

1. CHAPTER I

To find out alginate-degrading enzymes from commercially available enzymes with the gel diffusion assay developed by the author *et al.* (Yoshida *et al.*, 1997), to purify endo-poly(1,4- α -L-guluronide)lyase (EndoGase) from *Flavobacterium multivolumm*, and to investigate the characteristic of the purified enzyme.

2. CHAPTER II

To purify endo-poly(1,4- β -D-mannuronide)lyase (EndoMase) from Abalone Acetone Powder, and to investigate the characteristic of the purified enzyme.

3. CHAPTER III

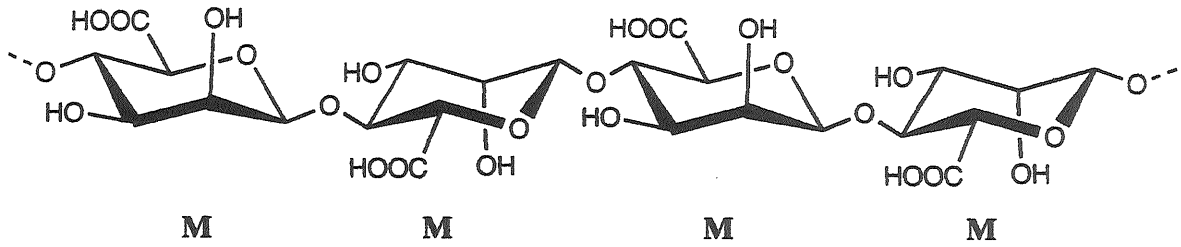
To purified endo-poly(1,4- β -D-mannuronide-1,4- α -L-guluronide)lyase (EndoMGase) from *Flavobacterium multivolum.*

4. CHAPTER IV

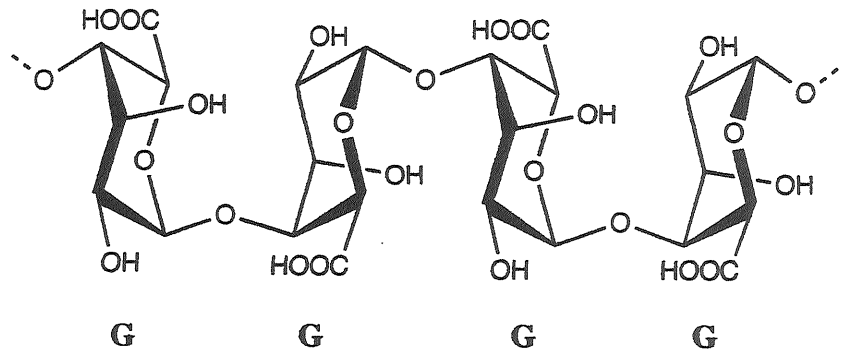
To develop a simple method for the preparation of M-block from alginate by applying the substrate specificity of partially purified EndoGase from *F. multivolum*, and to discuss on the comparison of my method with Haug's method.

5. CHAPTER V

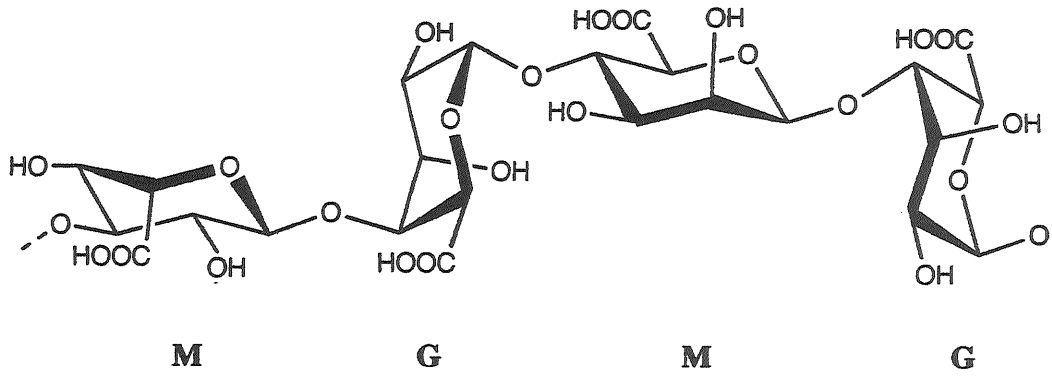
To clarify some properties of crude enzyme from *F. multivolum*, and to study the large scale preparation of oligo-uronide from alginate with the enzyme.



M-block (poly-mannuronate)



G-block (poly-guluronate)



MG-block (hetero-polymeric random structure)

Fig. GI-1. Partial Structures of M-block, G-block and MG-block in Alginate.
M, β-D-mannuronic acid; G, α-L-guluronic acid.

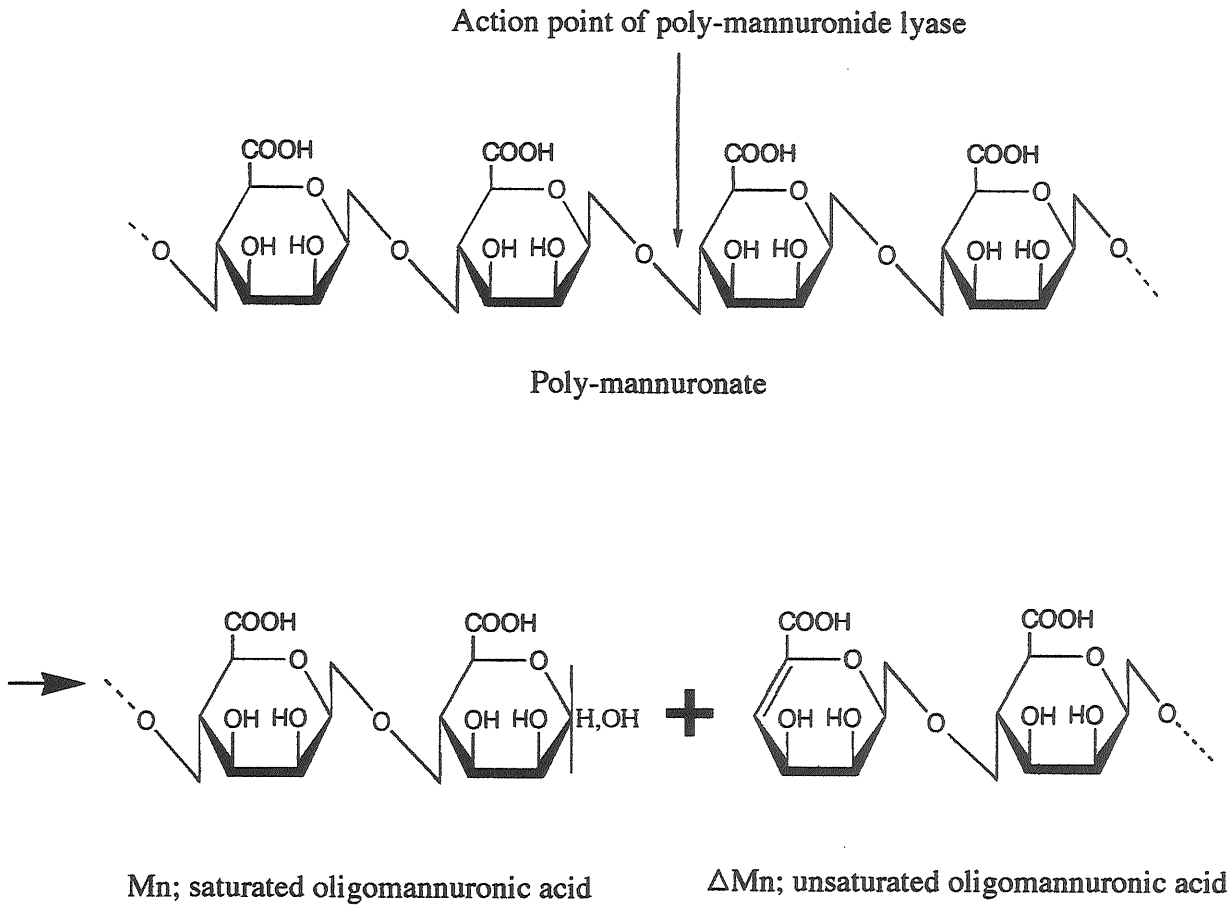


Fig. GI-2. Degradation Mechanism of Poly-mannuronate by Poly-mannuronide Lyase. M, mannuronic acid; G, guluronic acid; Δ, 4-deoxy-α-L-*erythro*-4-enopyranosyl-uronic acid

CHAPTER I

PURIFICATION AND CHARACTERIZATION OF ENDO-POLY(1,4-α-L-GULURONIDE) LYASE FROM *Flavobacterium multivolum*

SUMMARY

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℃, and was stable in the pH range of 6.0 to 9.0 and at temperatures up to 30℃. 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 an endo-poly(1,4-α-L-guluronide) lyase (EC 4.2.2.11).

INTRODUCTION

As described in the general introduction, alginate has an extremely intricate chemical structure. Thus, it is considered that various kinds of alginate-degrading enzymes, which have different substrate specificities for the degradation of the alginate,

exist in nature. Thus far, many sources, such as marine algae, marine molluscs and microorganisms, are known to produce alginate-degrading enzymes (Gacesa, 1992; Sutherland, 1995). However, the study on the characterization of the purified enzyme is a few, and the substrate specificity of the enzyme is hardly revealed. For this reasons, the author tried to find out an alginate-degrading enzyme (algonate lyase) from commercially crude enzyme, and obtained two enzyme sources, namely Multivolum enzyme and Abalone Acetone Powder. In this chapter, the author purified the alginate lyase originated from *Flavobacterium multivolum*, and investigated some properties of the purified enzyme.

MATERIALS AND METHODS

Screening test for alginate-degrading activity of commercial enzyme

Commercially available enzymes were used for the screening test of alginate-degrading activity. The enzyme were as follows; Deaminase (*Aspergillus meleus,* Amano Co.), Pectinase G (*Asp. pulrerulentus*, Amano Co.), Cellulase No. 22175 (*Humicola insolens*, Fuluka Co.), Meicelase P-1 (*Tricoderma viride*, Meiji Co.), Multivolum enzyme (*Flavobacterium multivolum* K-11, Nagase Biochemicals Ltd.), Pectinase Nagase (*Asp. Niger*, Nagase Biochemicals, Ltd.), Cellulase C-0901 (*Penicillium funiculosum*, Sigma Co.), Driselase D-954 (Irpex lacteus, Sigma Co.), Abalone Acetone Powder (Sigma Co.), and other commercial preparations. On the other hand, the screening test was carried out by the gel diffusion assay for alginate lyase (Yosida *et al.*, 1997). The assay method was as follows. An enzyme solution was put on a paper disk which was placed on an agar plate containing 1 % dabsyl-alginate,

and then the plate was incubated at 30℃ for 2 days. The enzyme having alginate-degrading activity formed a diffusion zone on the agar plate (Fig. Ⅰ-1). By this method, Multivolum enzyme and Abalone Acetone Powder were selected from the commercial preparations as two enzyme sources having strong alginate-degrading activity.

Enzyme and substrates

A crude powder of alginate-degrading enzyme, an extracellular enzyme originating from *F. multivolum* K-11, was purchased from Nagase Biochemicals, Ltd. (Fukuchiyama, Kyoto). Alginate of "Duck algin 350-M" (Kibun Food Chemifa Co., Ltd., Tokyo) was used in this experiment. The M/G ratio of this alginate was 0.94, obtained by the method of Haug *et al.* (1974). Three blocks, namely the G-block, M-block and MG-block, were prepared from the alginate, by the method of Haug *et al.* (1967). The M and G contents of the three were determined by CD analysis using a JASCO J-720 spectropolarimeter (JASCO corporation, Tokyo) according to the method of Morris *et al.* (1980). The result established that the G-block contained 89% G, the M-block contained 92% M, and the MG-block contained 57% M. D-Mannuronic acid lactone was purchased from Sigma Chemicals Co. (St. Louis, Mo.)

Assay for alginate lyase activity

For the enzyme assay, 0.5 ml of 1% solution of sodium alginate (M/G; 0.94), dissolved in 50 mM Tris-HCl buffer (pH 8.0) , was poured into a test tube and incubated at 37℃ 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℃ for 10 min to stop the reaction, and then 0.2 ml of the mixture was withdrawn to an another test tube. The amount of unsaturated material produced by the lyase action

was measured by the TBA reaction (Weissbach and Hurwitz, 1959; Preise and Ashwell, 1962).

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 A548 of 0.290 in the TBA reaction.

Protein measurement

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 is 1.0.

Purification of endo-poly(1,4-α-L-guluronide) lyase

All the procedures were done at about 4℃.

Preparation of crude enzyme solution A 12 g of sample 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 overnight.

CM-Toyopearl 650M column chromatography The dialyzed enzyme solution was centrifuged at 6,200 x *g*, and the supernatant (total activity; 1,850 units) was applied to a CM-Toyopearl 650M (Tosoh, Tokyo) column (26 x 400 mm) 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 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 number 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.

First chromatofocusing The lyophilized enzyme was dissolved in 10-fold diluted Polybuffer 96 (pH 7.0, Pharmacia, Uppsala, Sweden) and then loaded on the Polybuffer Exchanger 94 (the same) column (13 x 300 mm) 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.

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.

Ultrogel AcA-54 column chromatography In order to remove Polybuffer 96 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 (16 x 850 mm) 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 potions. The fractions of alginate-degrading enzyme, tube numbers 33 to 38 (total activity; 319 units) were combined.

Estimation of molecular mass and isoelectric point

The molecular mass of the enzyme was estimated under a denaturing condition by SDS-PAGE (Weber and Osborn, 1969). Molecular mass markers (low range, Bio-rad

Laboratories, Harcules, Calif.) were used as standard proteins. The gels were stained with Coomassie brilliant blue R-250. On the other hand, the molecular mass 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 x 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 mass marker kit (Oriental Yeast Co., Tokyo) was used as a standard marker for proteins.

The pI of the purified enzyme was measured by analytical isoelectric focusing (IEF) using a Multipore 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 in *vacuo* with 6N HCl at 110℃ for 24 h. The resultant amino acids were determined with an amino acid analyzer (type 835-50, Hitachi, Tokyo).

Thin-layer chromatography

To characterize the reaction products, after the enzyme reaction mixture was treated with cation-exchange resin (Amberlite IR-200), the mixture was put on a TLC plate of silica gel 60 (MERCK JAPAN LTD. Tokyo). The TLC plate was then developed with a solvent system of 1-butanol : formic acid : water (4 : 6 : 2, v/v) by the ascending method. The spots of the uronic acids were visualized by heating the TLC

plate at 150℃ for 5 min after spraying with concentrated sulfuric acid. The unsaturated sugars were also stained by TBA reagent (Warren, 1960; Takeuchi *et al.*, 1994; Nibu *et al.*, 1995).

Substrate specificity

To examine the specificity of the enzyme, a 100 μl of enzyme solution containing 2.1 units was added to each 1.0 ml of 1% solution of G-, MG- and M-block, and the reaction was then continued at pH 8.0 and 37℃. At certain intervals of time (0, 0.25, 0.5, 1, 3, 6 and 12 h), a 100 μl of the reaction mixture was taken out and immediately heated at 100℃ for 5 min to inactivate the enzyme. The unsaturated uronic acids produced by the enzyme reaction were determined by the TBA reaction (Weissbach and Herwitz, 1959; Preiss and Ashwell, 1962). On the other hand, the reaction mixture was subjected to TLC for the characterization of the reaction products. The TLC was done as described above.

RESULTS AND DISCUSSION

Purification of endo-poly(1,4-α -L-guluronide) lyase

The result of the purification procedure is summarized in Table I -1. For purification of alginate lyase from *F. multivolum* K-11, the crude enzyme was subjected to sequential CM-TOYOPEARL, chromatofocusing (twice), and Ultrogel AcA-54 column chromatographies. The purified enzyme revealed a single band on SDS-PAGE (Fig. I -2). 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.

Molecular mass and isoelectric point of endo-poly(1,4-α -L-guluronide) lyase

The molecular mass of the enzyme was estimated to 41-kDa by HPLC gel filtration analysis (data not shown) and 43-kDa by SDS-PAGE (Fig. I -2), indicating that the enzyme was a single peptide. The 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 lyase from *Enterobacter cloacae* M-1 (Nibu *et al.*, 1995), *Klebsiella aerogenes* (Lange *et al.*, 1989) and an unidentified bacteria (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 enzyme from *F. multivolum* was estimated to be 8.7 (Fig. I -3). The pI of the enzyme was very close to those of the guluronate lyases from *E. 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).

Amino acid composition of endo-poly(1,4-α -L-guluronide) lyase

The amino acid composition of the enzyme is shown in Table I -2. The 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 %.

General properties of endo-poly(1,4-α -L-guluronide) lyase

The effects of pH and temperature on purified enzyme are shown in Figures I -4A and 4B, respectively. The optimum pH for the activity was around 8.0 (Fig. I -4A), and the optimum temperature for the activity was around 40℃ (Fig. I -4B). The optimum pH of my 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 my enzyme was similar to that from *K. aerogenes* (Lange *et al.*, 1989) but not to those of two guluronate lyases (Takeshita *et al.*, 1993; Nibu *et al.*, 1995). On the other hand, the effects of pH and temperature on the stability of the purified enzyme are shown in Figures I -4C and 4D, respectively. The enzyme was stable between pH 6.0 and pH 9.0 (Fig. I -4C). The enzyme was stable up to 30℃, but no enzyme activity was obtained after the treatment at 50℃ for 2 h (Fig. I -4D). This enzyme was more thermostable than the guluronate lyase from *Enterobacter cloacae* M-1 (Nibu *et al.*, 1995) but was more unstable more than the marine bacterial guluronate lyase (Takeshita *et al.*, 1993).

Effects of chemical compounds on endo-poly(1,4-α -L-guluronide) lyase

Table I -3 shows the effects of chemical compounds on the purified enzyme. The enzyme activity was decreased by the addition of EDTA, PCMB, MIA, TNBS, and NBS, while treatments with SDS, *N*-ethylmaleimide and 2-mercaptoethanol were found to have no significant effect on the enzyme activity. The result suggests that this enzyme was sensitive toward EDTA, and amino acid residues containing SH-group and triptophan residue of the enzyme participated in enzyme activity. On the other hand, the activity of EDTA-treated enzyme was remarkably restored by treatment with various kinds of metal compounds, especially $MnCl_2$, $FeCl_3$, etc. (Table I -4). This effect indicates that the enzyme required some metal compounds for maximal activity as well

as the guluronate lyase from *E. cloacae* M-1 with the notable exception especially the case of Fe Cl_3 (Nibu *et al.*, 1995).

Substrate specificity of endo-poly(1,4-α -L-guluronide) lyase

Figure I -5 shows the time-course of EndoGase reaction followed by the TBA reaction (Fig. I -5A) and TLC (Fig. I -5B) toward M-, MG- and G-blocks. The increase in the absorbance at 548 nm was rapid during the beginning of the reaction but gradually leveled off thereafter, although the enzyme degraded the three blocks in the order of G-block (G content; 89 %) > MG-block (M content; 57 %) >> M-block (M content; 92 %) (Fig. I -5A). Subsequently, to detect the enzymatic degradation products, the author performed TLC with the reaction mixture (Fig. I -5B). Several kinds of oligomers were explicitly observed from the G- and MG-blocks with significant difference but not from M-block. The main products were five or six kinds of oligomers from the MG-block (MG-block in Fig. I -5B), whereas there were three kinds of oligomers from the G-block (G-block in Fig. I -5B). These findings suggest that this enzyme was classified as an endo-poly(1,4- α -L-guluronide) lyase (EC 4.2.2.11).

In this chapter, the author purified an alginate-degrading enzyme from *F. multivolum* K-11 to electrophoretic homogenity and revealed some properties of the enzyme. The purified enzyme was classified as an endo-poly(1,4- α -L-guluronide) lyase (EC 4.2.2.11) based on its substrate specificity. However, the author 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 (CHAPTER V) and the purified enzyme. Therefore, the author will study the purification and characterization of another enzyme from *F. multivolum* K-11, and the

details will be described in CHAPTER Ⅲ.

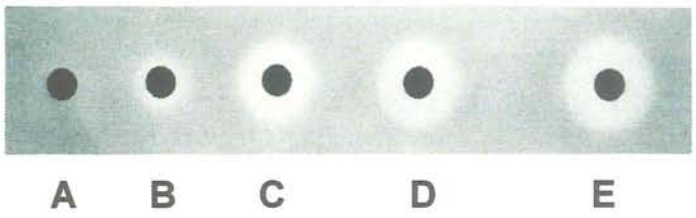


Fig. I -1. Gel Diffusion Assay for Alginate Lyase.

A paper disk (ø 6 mm) was placed on an agar plate (0.5 mm thickness) containing 1 % dabsyl-alginate, and then an enzyme solution (10 μ l) of *Flavobacterium* alginate lyase was put on the desk. After incubation at 30 ℃ for 2 days, the diffusion zone obtained by the enzymatic degradation of dabsyl-alginate was observed. A, blank; B, 1.05 milliunits: C, 10.5 milliunits; E, 42 milliunits.

Table I -1. Summary of the Purification of EndoGase from *Flavobacterium multivolumn* 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

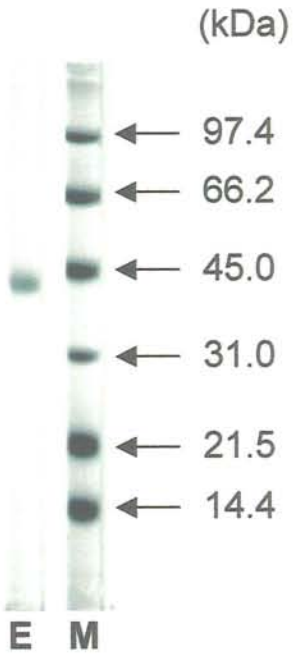


Fig. I -2. Analysis of the Purified Enzyme by SDS-PAGE and Coomassie Brilliant Blue R-250 staining.
Lane E, purified enzyme; lane M, relative molecular mass (kDa) markers.

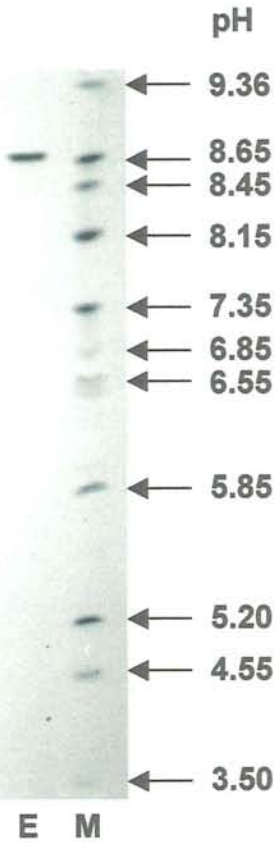


Fig. Ⅰ-3. Estimation of pI of Purified Enzyme by Isoelectric Focusing. Lane M, pI standards; lane E, purified enzyme.

Table Ⅰ-2. Amino Acid Composition of EndoGase

Amino acid	Mol percentage (%)
Asx	12.4
Thr	10.2
Ser	12.2
Glx	7.1
Gly	15.2
Ala	5.9
Val	8.9
Ile	5.6
Leu	8.0
Tyr	0.7
Phe	3.3
Lys	6.7
His	1.0
Arg	2.9

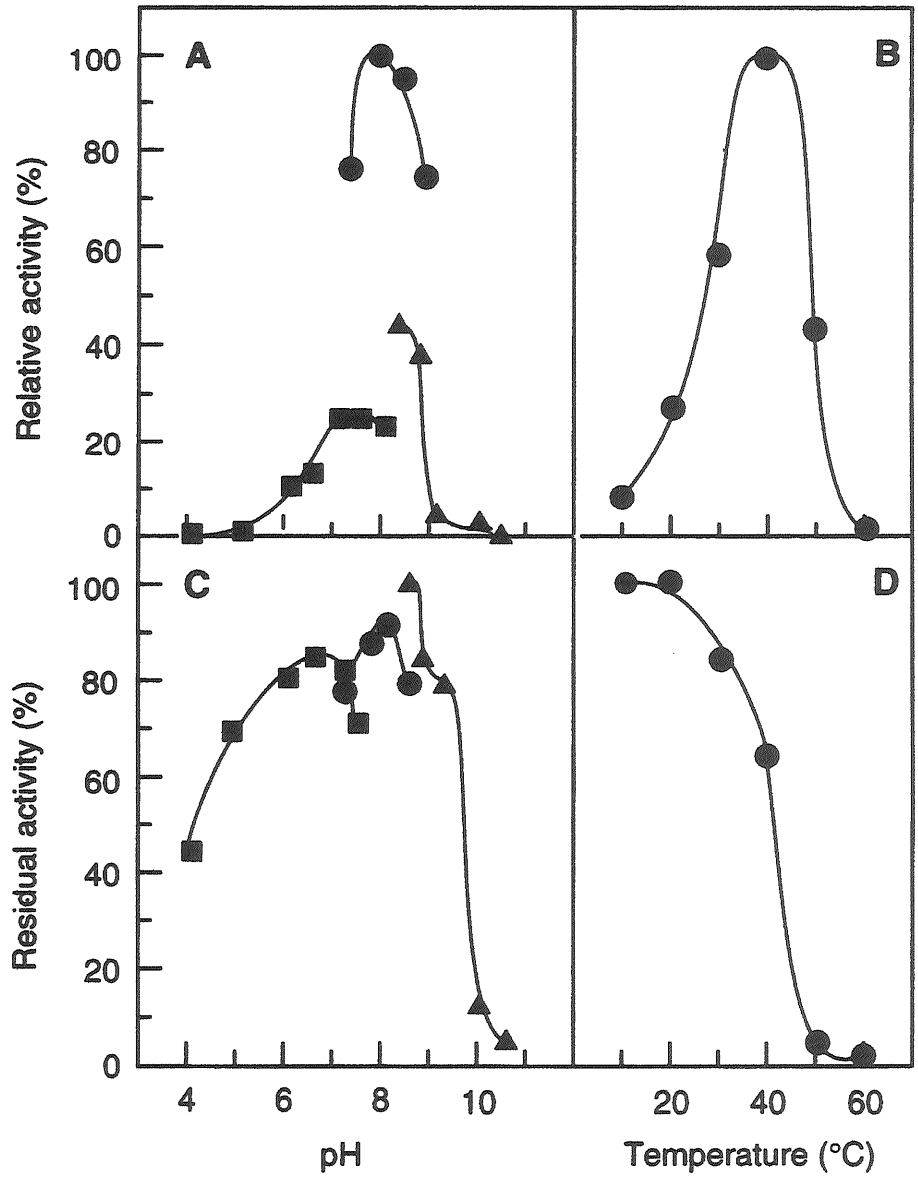


Fig. I -4. Enzymatic Properties of Purified Enzyme.

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℃ 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. Enzyme solutions were incubated at various pH values, using McIlvaine buffer (■), Tris-HCl buffer (●), and Atkins-Pantin buffer (▲), at 25 ℃ 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 stability was incubated at various temperature at pH 8.0 in Tris-HCl buffer for 1 h, and residual activity was determined.

Table I -3. Effects of Chemical Compounds on EndoGase

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 compounds (1×10^{-3} M) was pre-incubated in Tris-HCl buffer at pH 8.0 and 25℃ for 15 min, the residual activity was assayed. The residual activities are expressed as the percentage of the activity in the absence of the test compound. EDTA, ethylendiamintetraacetic acid; SDS, sodium dodecyl sulfate; PCMB, *p*-chloromercuribenzoic acid; MIA, monoiodoacetic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

Table I -4. Restoration of EDTA-treated EndoGase by Metal Compounds

Compound	Relative activity (%)
Control	100
EDTA	13
+ $NiCl_2$	73
+ $MnCl_2$	146
+ $CaCl_2$	116
+ $CdCl_2$	35
+ $CuCl_2$	82
+ $PbCl_2$	82
+ $ZnCl_2$	36
+ $AlCl_3$	91
+ $HgCl_2$	9
+ $FeSO_4$	99
+ $FeCl_3$	120
+ $BaCl_2$	4
+ $MgCl_2$	71
+ $CoCl_2$	73

The enzyme solution containing EDTA 1 mM (final concentration) was incubated in Tris-HCl buffer at pH 8.0 and 25℃ for 15 min. Then, each of the metal compounds was added to EDTA-treated enzyme solution to bring to 2 mM of final concentration, and the mixture was incubated at 25℃ for 15 min. After incubation, the enzyme activity was assayed. Enzyme activity was expressed as percentage of the activity in the absence of EDTA and metal compounds.

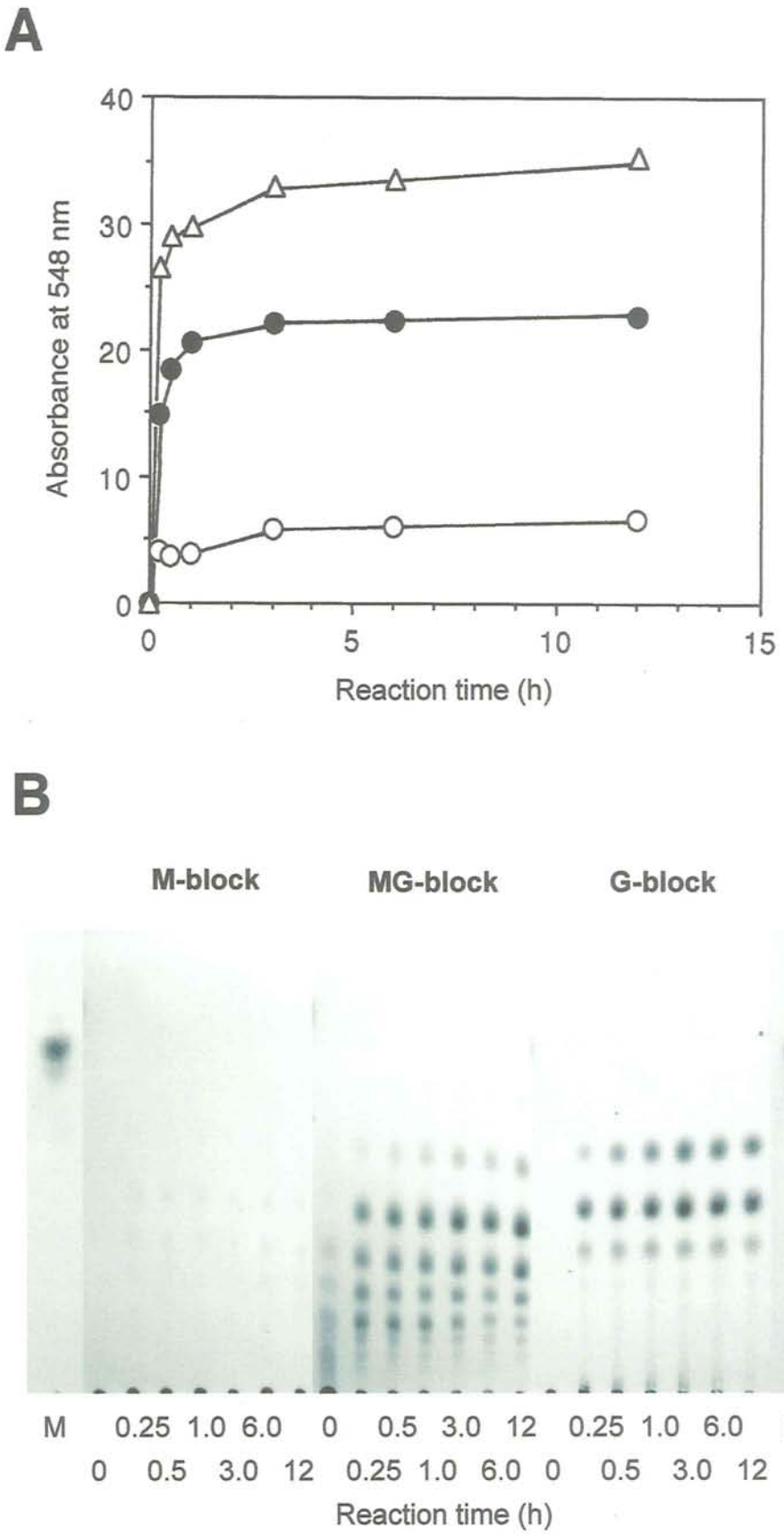


Fig. I -5. Time-course of the Enzyme Reaction toward G-, MG- and M-blocks. A: The course of enzyme reaction followed by TBA reaction. △, G-block; ●, MG-block; ○, M-block. B; The course of enzyme reaction followed by TLC staining with sulfuric acid. Lane M, D-mannuronic acid lactone.

CHAPTER Ⅱ

PURIFICATION AND CHARACTERIZATION OF ENDO-POLY(1,4-β-D-MANNURONIDE) LYASE FROM ABALONE ACETONE POWDER

SUMMARY

An alginate lyase was obtained from Abalone Acetone Powder (commercial enzyme) by successive chromatographies on DEAE-Toyopearl 650M, 1st Butyl-Toyopearl 650S, 2nd Butyl-Toypearl 650S, and Ultrogel AcA-54. The purified enzyme gave a single band on SDS-PAGE, and had a molecular mass of 32-kDa by SDS-PAGE and 30-kDa by gel filtration. The optimum pH and temperature for enzyme activity were pH 7.5-9.0 and 30℃, respectively. The enzyme was stable from pH 4 to 8 and at temperature below 30℃. The additions of PCMB, MIA, NEM and TNBS decreased the enzyme activity. Some divalent cations such as Mn^{2+}, Ca^{2+} and Mg^{2+} had stimulating effects on the activity, but Ni^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+} and Fe^{3+} inhibited the enzyme activity. The enzyme degraded M-block more than MG-block, but did not act on G-block. Moreover, the enzyme produced various DPs of oligouronic acids having 4,5-unsaturated sugar from M-block. Thus, the results suggested that the purified enzyme was classified as an endo-poly(1,4-β-D-mannuronide) lyase (EC 4.2.2.3).

INTRODUCTION

The author assumed in the general introduction of my thesis that there are various

kinds of alginate-degrading enzymes indicating different substrate specificities, because the chemical structure of alginate is very complicated.

In CHAPTER I , the author attempted to found out one of alginate-degrading enzymes from crude enzyme preparation of *Flavobacterium multivolum*, and obtained an enzyme in pure state from the enzyme preparation. From the characteristic of enzyme, the enzyme was specific for polyguluronate (G-block), namely endo-poly(1,4-α-L-guluronide) lyase.

Next, the author aimed to obtain endo-poly(1,4-β-D-mannuronide) lyase (Endo-Mase) from other enzyme preparation. It is known to include EndoMase (Nakada *et al.*, 1967) in Abalone Acetone Powder, but various properties of the enzyme, especially substrate specificity, are not known. From this reason, CHAPTER II dealt with the purification and characteristic of EndoMase from Abalone Acetone Powder, and the substrate specificity of the enzyme toward the block structures of alginate.

MATERIALS AND METHODS

Enzyme and substrates

Enzyme, Abalone Acetone Powder, was purchased from Sigma Chemical Co. (St. Louis, Mo). Alginate, "Duck algin 350M-T" described in CHAPTER I , was used in the study. G-, M- and MG-blocks were the same as that described in CHAPTER I . D-mannuronic acid lactone was purchased from Sigma Chemical Co. (St. Louis, Mo).

Assay for alginate lyase activity

Enzyme activity was determined at the conditions of pH 8.0 and 37℃ for 30 min by the method described in CHAPTER I .

Protein measurement

The protein concentration in the purification process was determined using BCA Protein Assay Reagent (Pierce Co. IL.) with albumin from Bovine A4387 (Sigma Chemicals Co. Mo.) as a standard protein.

Purification of endo-poly (1,4-β -D-mannuronide) lyase

Preparation of crude enzyme solution A 5 g of Abalone Acetone Powder was suspended in 150 ml of 0.1 M phosphate buffer (pH 7.0) and slowly stirred for 90 min. The supernatant was dialyzed against 10 mM phosphate buffer (pH 7.0), and the enzyme solution, thus obtained, contained 677 units as total activity.

DEAE-Toyoperl 650M column chromatography The dialyzed enzyme solution was applied to a DEAE-Toyopearl (Tohso, Tokyo) column (26 x 400 mm) equilibrated with 10 mM phosphate buffer (pH 7.0). After the column was washed with about 600 ml of the same buffer, the enzyme in the column was eluted 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 potions. The enzyme having alginete-degrading activity separated into three fractions, namely non-adsorbed fraction (total activity; 426 units), tube numbers 21 to 40 (total activity; 24.7 units) and tube numbers 41 to 56 (total activity; 23.8 units). A 1,040 ml of the non-adsorbed fraction was concentrated to 250 ml by ultra-filtration using a YM-3 membrane (Amicon, Bevery, Mass.).

First Butyl-Toyopearl 650S column chromatography A 60.75 g of ammonium sulfate was slowly added with stirring to the 250 ml of concentrated enzyme solution obtained above. The enzyme solution was then applied to a Butyl-TOYOPEARL 650S (Tohso, Tokyo) column (30 x 150 mm) equilibrated with 10 mM phosphate buffer (pH 7.0) containing 40 % saturated ammonium sulfate. After the column was washed with about 300 ml of the same buffer, the enzyme in the column was eluted a linear gradient from 40% to 0% saturated ammonium sulfate at a flow rate of 72 ml/h. The eluate was fractionated into 6-ml potions. The fraction having lyase activity, tube numbers 44 to 58 (total activity; 175 units), was dialyzed against 10 mM phosphate buffer (pH 7.0).

Second Butyl-Toyopearl 650S column chromatography A 36.45 g of ammonium sulfate was added with stirring to the 150 ml of dialyzed enzyme solution. The enzyme solution was applied to a Butyl-Toyopearl 650S column (16 x 200 mm) and chromatographed by the method described above. The flow rate was 20 ml/h, and the eluate was fractionated into 2-ml portions. The fraction having lyase activity, tube numbers 20 to 40 (total activity; 110 units), was concentrated to 5.5 ml by ultra-filtration with a YM-3 membrane.

Ultrogel AcA-54 column chromatography In order to further purify the enzyme, the concentrated enzyme solution was applied to the column (16 x 850 mm) of Ultrogel AcA-54 (LKB) equilibrated with 0.2 M phosphate buffer. The elution was done at a flow rate of 15 ml/h, and the eluate was fractionated into 3-ml portions. The fraction showing lyase activity, tube numbers 38 to 41 (total activity; 69.2 units) was pooled.

Estimation of molecular mass

The molecular mass of purified enzyme was estimated by SDS-PAGE (Laemmli, 1970), and also estimated by gel filtration as described in CHAPTER Ⅰ.

Thin-layer chromatography

TLC was done by the method described in CHAPTER Ⅰ.

Substrate specificity of endo-poly(1,4-β -D-mannuronide) lyase

The enzyme was incubated with either M-block, G-block or MG-block to elucidate the substrate specificity. One ml of 1% solution of each substrate dissolved in Tris-HCl buffer (pH8.0) was pre-incubated at 37℃ for 15 min. A 100 μl of enzyme solution containing 2.0 units was added to the substrate solution, and the reaction was continued at pH 8.0 and 37℃. A 100 μl of the reaction mixture was withdrawn at the described time (0, 0.25, 0.5, 1, 3, 6 and 12 h), and heated at 100℃ for 5 min to inactivate the enzyme. Unsaturated uronic acids in the reaction mixture were determined by TBA method (Weissbach and Hurwitz, 1959; Preiss and Ashwell, 1962), and the reaction products were analyzed by TLC described in CHAPTER Ⅰ.

RESULTS AND DISCUSSION

Purification of endo-poly(1,4-β -D-mannuronide) lyase

Endo-poly (1,4-β -D-mannuronide) lyase (EndoMase) was purified from Abalone Acetone Powder by the column chromatographies of DEAE-Toyopearl 650M, first and second Buthyl-Toyopearl 650S, and Ultrogel AcA-54. The result of enzyme purification is summarized in Table Ⅱ-1. By these procedures, EndoMase was purified 47.6-fold with an activity yield of 10.2% from the crude extract of Abalone Acetone Powder. The

purified EndoMase gave a single band on SDS-PAGE (Fig. Ⅱ-1).

Molecular mass of endo-poly(1,4-β -D-mannuronide) lyase

The relative molecular mass of EndoMase was 32-kDa estimated by SDS-PAGE (Fig. Ⅱ-1), and 30-kDa estimated by gel filtration (Fig. Ⅱ-2). The results indicate that the native EndoMase of Abalone existed as a single polypeptide protein. Recently, Shimokawa *et al.* (1997) have reported that *Dendryphiella salina* IFO 32139 produced an extracellular EndoMase. The purified EndoMase (Shimokawa *et al.*, 1997) had a molecular mass of 35-kDa by SDS-PAGE and 36-kDa by gel filtration. Thus, the molecular mass of EndoMase from Abalone was slightly smaller than that of EndoMase from *D. salina.*

Effects of pH and temperature on endo-poly(1,4-β -D-mannuronide) lyase

Figure Ⅱ-3 shows the effects of pH and temperature on the enzyme activity and stability. The optimal pH and the optimal temperature for activity were 7.5-9.0 (Fig. Ⅱ-3A) and 30℃ (Fig. Ⅱ-3B), respectively. On the other hand, the EndoMase was stable over the pH range 4-8 (Fig. Ⅱ-3C), and at temperature up to 30℃ (Fig. Ⅱ-3D).

Effects of chemical compounds on endo-poly(1,4-β -D-mannuronide) lyase

The experimental conditions are described in the legend of Table Ⅱ-2. The table shows the effects of chemical reagents and metal ions on EndoMase activity. The enzyme activity was decreased by the addition of PCMB, MIA, NEM and TNBS. The results suggest that this enzyme may be sensitive to amino acid residues containing SH-group of cysteine and imidazole-group of histidine because above reagents are known as a SH-reagent. NBS completely inhibited the enzyme activity. The inhibition by NBS

suggests that tryptophan residues may play an important role at the active site or the substrate binding site of the enzyme. The inhibition caused by chemical modification of tryptophan residues has been reported on polyguluronate lyases from *Klebsiella pneumoniae* (Hicks and Gascesa, 1994) and polymannuronate lyase from *Turbo cornutus* (Muramatsu and Egawa, 1982). On the other hand, the treatment with EDTA was not affected very much. Among the tested cations, some of divalent metal ions such as Mn^{2+}, Ca^{2+}, and Mg^{2+} had stimulating effect on the activity, but Ni^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+}, Fe^{2+} and Fe^{3+} inhibited the enzyme activity.

Substrate specificity of endo-poly(1,4-β -D-mannuronide) lyase

Figure Ⅱ-4 shows the course of the enzyme reaction on M-block, MG-block and G-block. When the enzyme acted on M- and MG-blocks, the absorbance at 548 nm rapidly increased during the beginning of the reaction, but the increase in the absorbance immediately leveled off thereafter. Furthermore, the enzyme degraded M-block more than MG-block but did not act on G-block (Fig. Ⅱ-4A). On the other hand, 4,5-unsaturated oligosaccharides having various DPs were produced from M-block by enzyme reaction, and di-, tri- and tetra-uronic acids were accumulated by further degradation (M-block in Fig. Ⅱ-4B). However, degradation product was not detected from G-block (G-block in Fig. Ⅱ-4B). It seems that even if EndoMase from Abalone Acetone Powder is specific to M-block, the enzyme also acts on MG-block. Because MG-block used in this experiment is composed of 57% M. As the conclusion, the above results suggest that the alginate lyase from Abalone Acetone Powder was a kind of poly(1,4-β -D-mannuronide) lyase (EC 4.2.2.3).

Nakada and Sweeny (1967) reported the presence of two alginases (alginases I

and Ⅱ) in abalone hepatopancreas. One of them alginase Ⅰ, was specific for 1,4-β-D-mannuronic acid units. The optimum pH for the enzyme reaction of alginase Ⅰ was between 7.4 and 7.6, and divalent metal ions such as Mg^{2+} and Mn^{2+} had stimulating effect on the activity of alginase Ⅰ. The above properties were similar to that of EndoMase obtained by the author. However, it cannot be judged whether alginase Ⅰ is identical to EndoMase because the detailed characteristics of alginase Ⅰ were not studied.

Table Ⅱ-1. Summary of the Purification of EndoMase from Abalone Acetone Powder

Purification step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification (-fold)
Crude enzyme solution	805	667	0.83	100	1.00
DEAE-Toyopearl 650M	172	426	2.48	63.0	2.95
First Butyl-Toyopearl 650S	9.93	175	17.6	26.0	21.0
Second Butyl-Toyopearl 650S	2.90	110	37.9	22.0	45.1
Ultrogel AcA-54	1.73	69.2	40.0	10.2	47.6

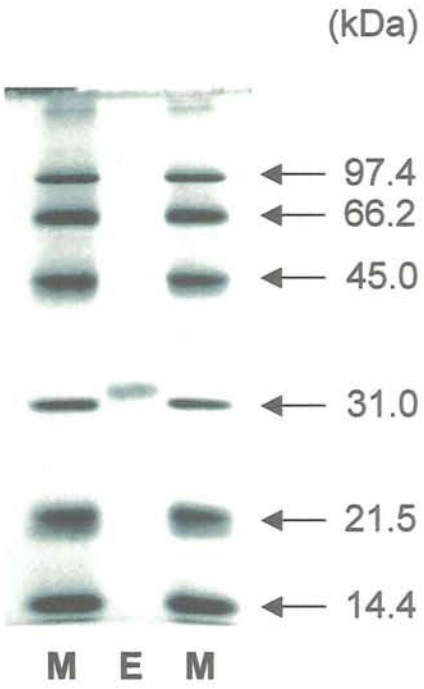


Fig. Ⅱ-1. Analysis of EndoMase by SDS-PAGE and Coomassie Brilliant Blue R-250 Staining. Lane E, purified enzyme; lane M, relative molecular mass (kDa) markers.

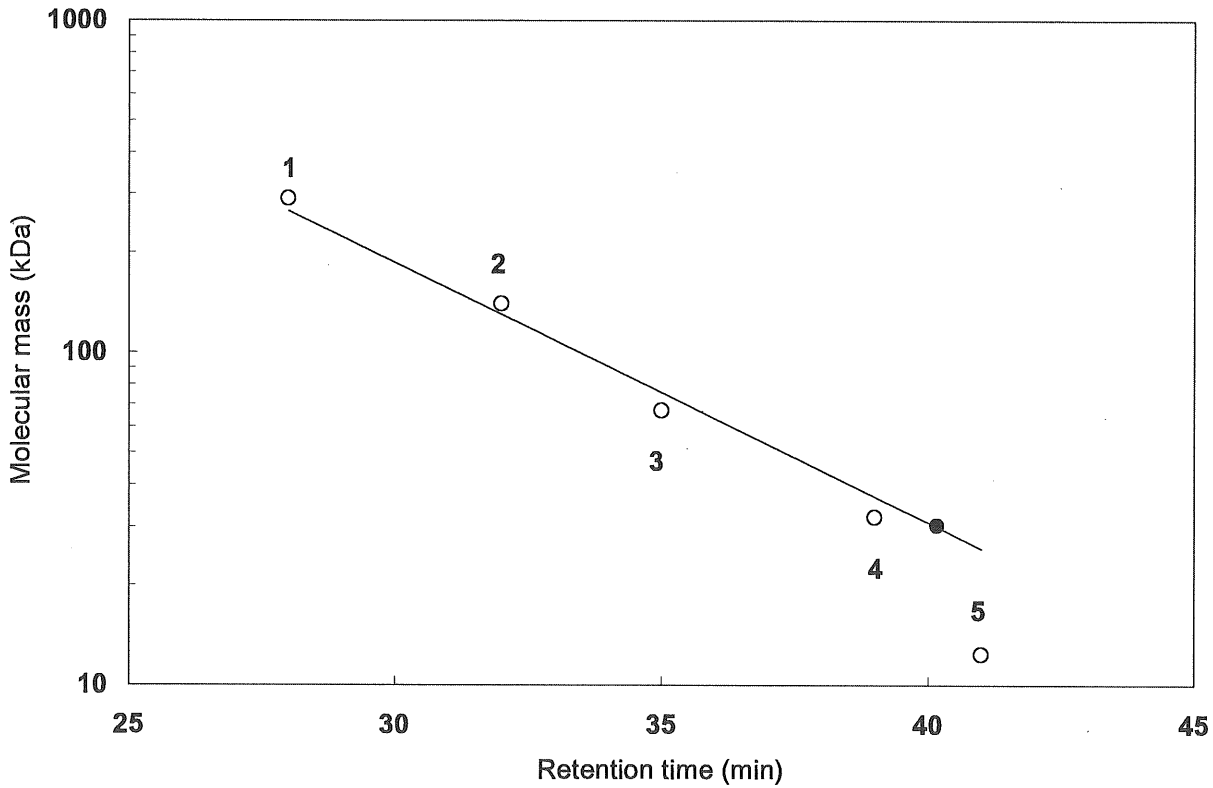


Fig. Ⅱ-2. Estimation of Molecular Mass of EndoMase by Gel Filtration Column Chromatography.
○, molecular mass standards (Oriental Co.): 1, yeast glutamate dehydrogenase (290,000); 2, pig heart lactate dehydrogenase (140,000); 3, yeast enolase (67,000); 4, yeast adenylate kinase (32,000); 5, horse heart cytochrome C (12,400); ●, purified EndoMase.

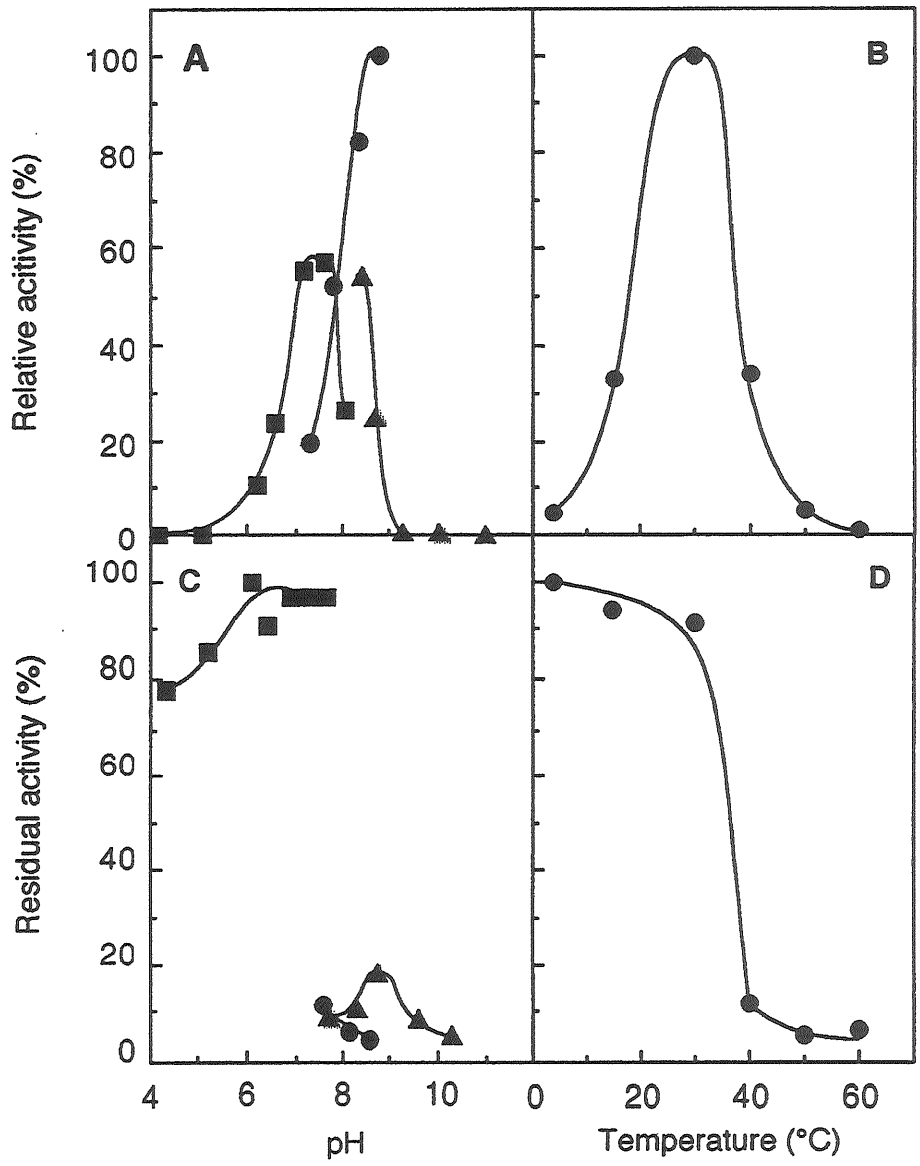


Fig. II-3. Enzymatic Properties of Purified Enzyme.

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℃ 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. Enzyme solutions were incubated at various pH values, using McIlvaine buffer (■), Tris-HCl buffer (●), and Atkins-Pantin buffer (▲), at 25℃ 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 stability was incubated at various temperature at pH 8.0 in Tris-HCl buffer for 1 h, and the residual activity was determined.

Table Ⅱ-2. Effects of Various Compounds on EndoMase

Compound	Residual activity (%)
None	100
EDTA	102
SDS	6
PCMB	57
MIA	10
N-Ethylmaleimide	28
2-Mercaptoethanol	232
TNBS	10
N-Bromosuccinimide	0
$NiCl_2$	4
$MnCl_2$	231
$CaCl_2$	170
$CdCl_2$	5
$CuCl_2$	0
$PbCl_2$	0
$ZnCl_2$	0
$AlCl_3$	44
$HgCl_2$	1
$FeSO_4$	13
$FeCl_3$	28
$BaCl_2$	82
$MgCl_2$	180
$CoCl_2$	128

After the enzyme solution containing a test compounds (1×10^{-3} M) was pre-incubated in Tris-HCl buffer at pH 7.5 and 25℃ 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, ethylendiamintetraacetic acid; SDS, sodium dodecyl sulfate; PCMB, *p*-chloromercuribenzoic acid; MIA, monoiodoacetic acid; TNBS, 2,4,6-trinitrobenzenesulonic acid.

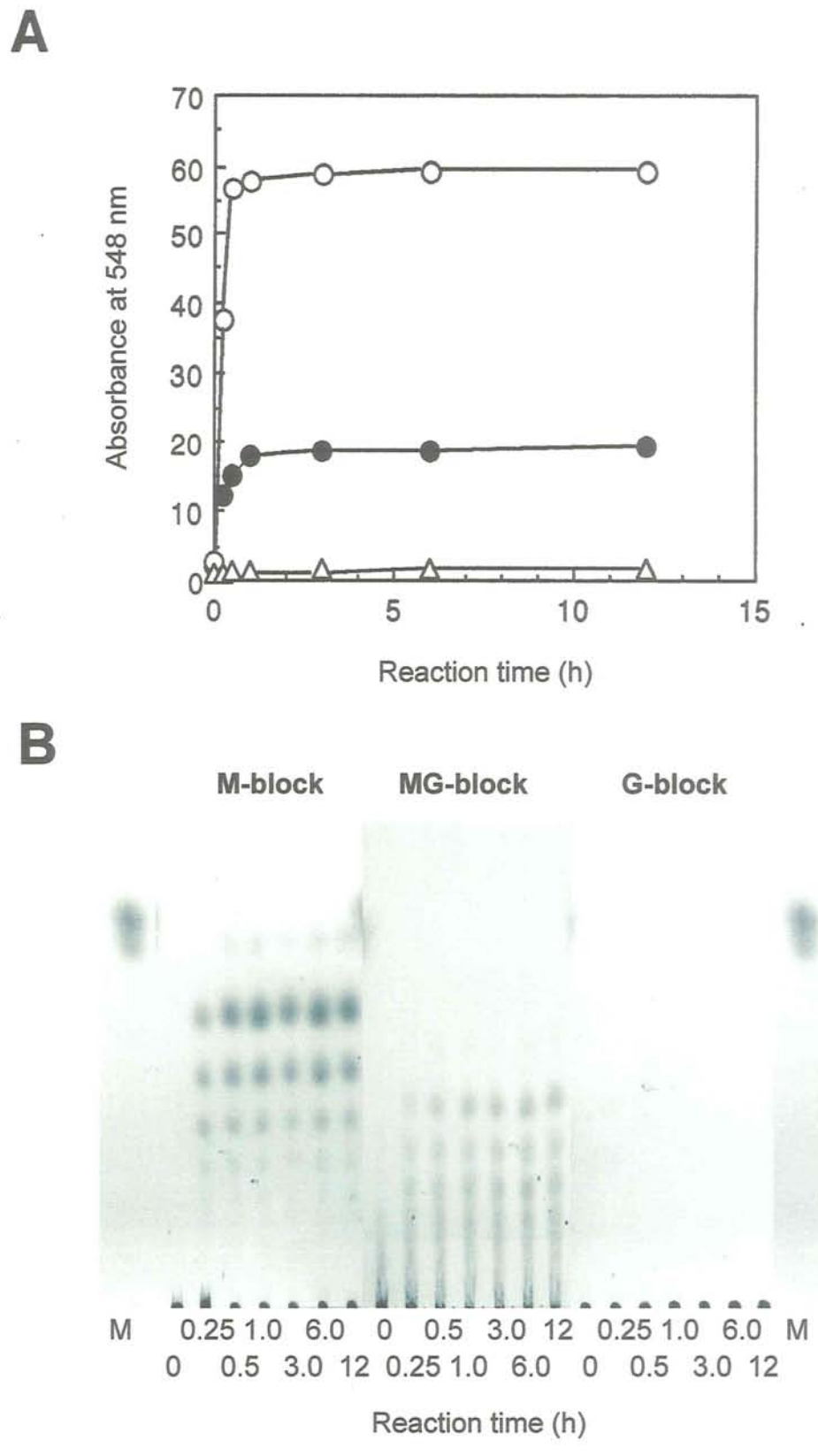


Fig. Ⅱ-4. Time-course of the Enzyme Reaction toward G-, MG- and M-blocks.
A: The course of enzyme reaction followed by TBA reaction. △, G-block;
●, MG-block; ○, M-block.
B: The course of enzyme reaction followed by TLC staining with sulfuric acid.
Lane M, D-mannuronic acid lactone.

CHAPTER Ⅲ

PURIFICATION AND CHARACTERIZATION OF ENDO-POLY(1,4-β-D-MANNURONIDE-1,4-α-L-GULURONIDE) LYASE FROM *Flabobacterium multivolum*

SUMMARY

A novel alginate lyase, designated the enzyme as an endo-poly(1,4-β-D-mannuronide-1,4-α-L-guluronide) lyase, was isolated from an extracllular enzyme 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 mass of the enzyme was 32-kDa by SDS-PAGE and 33-kDa 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℃, and was stable between pH 6.0 and 9.0, at temperature up to 20℃. The enzyme activity was remarkably inhibited by chemical compounds such as SDS, MIA and NBS, while EDTA and PCMB had no effect on the enzyme activity. The enzyme decomposed both the G-block (G content; 89 %) and the M-block (M content; 92 %) at nearly equal rates, and produced several kinds of unsaturated oligomers. Thus, the results suggested that the enzyme was classified as an endo-poly(1,4-β-D-mannuronide-1,4-α-L-guluronide) lyase (EndoMGase).

INTRODUCTION

The author obtained EndoGase from *Flavobacterium multivolum* in CHAPTER I and EndoMase from Abalone Acetone Powder in CHAPTER II. However, the author suspected that the crude enzyme of *F. multivolum* contained another alginate lyase except for EndoGase. This possibility was supported by the purification steps described in CHAPTER I, and by the difference in the optimum pH on the enzyme reaction between the purified enzyme and the crude enzyme (CHAPTER V). In addition, the crude enzyme almost completely degraded alginate, and produced several kinds of unsaturated oligosaccharides consiting of M, G and both (CHAPTER V). Thus, the author presumed that the crude enzyme consisted of more than one alginate lyase. In this connection, the author attempted to search another one.

In this chapter, the author fractionate the crude enzyme of *F. multivolum* and investigate 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 and substrates

Enzyme originated from *Flavobacterium multivolum*, alginate and three blocks (G-, M- and MG-blocks) were the same as that described in CHAPTER I.

Assay for alginate lyase activity

Enzyme activity was essentially assayed as described in CHAPTER I , under pH 8 and 37℃ for 5 min.

Protein measurement

The concentration was determined as described in CHAPTER I .

Purification of endo-poly(1,4-β -D-mannuronide-1,4-α -L-guluronide) lyase

Preparation of crude enzyme solution The preparation was done as described in CHAPTER I .

CM-Toyopearl 650M column chromatography The column chromatography was done with the same way described CHAPTER I , and the enzyme thus obtained was 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 (13 x 300 mm) 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, and then applied to a column (16 x 850 mm) of Ultrogel AcA-54 (LKB, Bromma, Sweden) that was pre-equilibrated with 40 mM phosphate buffer (pH 6.7) containing 0.24 M NaCl. The elution was done at a flow rate of 15 ml/ h. The eluate was fractionated into 3-ml aliquots. The fraction of EndoMGase was pooled.

Estimations of molecular mass and isoelectric point

The estimations were performed as described in CHAPTER I .

Amino acid analysis

The amino acids were determined by the method described CHAPTER I .

Substrate specificity

A 0.1 ml of the purified EndoMGase solution (2.2 units) was added to each 1.0 ml of a 1% solution of G-block, the MG-block and M-block preparations. The enzyme reaction was then performed at pH 8 and 37℃. 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℃ for 5 min to inactivate the enzyme. The unsaturated uronic acids produced by the enzyme reaction were determined by TBA reaction (Weissbach and Hurwitz, 1959; Preiss and Ashwell, 1962). On the other hand, the reaction mixture was subjected to TLC for the characterization of the reaction products. The TLC was performed as described in CHAPTER I .

RESULTS AND DISCUSSION

Purification of endo-poly(1,4-β -D-mannuronide-1,4-α -L-guluronide) lyase

The enzyme solution (total activity; 1,850 units) dialyzed against 1 mM phosphate buffer (pH 6.3) was applied to a CM-Toyopearl 650M column. Most of 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 tube numbers 19 to 25

(total activity; 838 units) containing EndoGase (Ochi *et al.*, 1995; Takeuchi *et al.*, 1997) and tube numbers 39 to 46 (total activity; 454 units). For further purification, the latter fraction 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 Excanger 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), was 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 Ⅲ-1). The purified enzyme could be resolved as a single band on both SDS-PAGE (Fig. Ⅲ-1) and IEF (Fig. Ⅲ-2). In addition, the author, at this time, suggest endo-poly(1,4- β -D-mannuronide-1,4- α -L-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 mass and isoelectric point of endo-poly(1,4-β -D-mannuronide-1,4-α -L -guluronide) lyase

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

condition was 8.2 (Fig. Ⅲ-2).

Amino acid composition of endo-poly(1,4-β-D-mannuronide-1,4-α-L-guluronide) lyase

Table Ⅲ-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 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 endo-poly(1,4-β -D-mannuronide-1,4-α -L-guluronide) lyase

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

Effects of chemical compounds on endo-poly(1,4-β -D-mannuronide-1,4-α -L -guluronide) lyase

Table Ⅲ-3 shows the effects of chemical compounds on the enzyme. The enzyme activity was decreased by the addition of SDS, MIA, TNBS and NBS. The result suggests that this enzyme activity is attributed to the amino acid residues containing the SH-group and the tryptphan 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. In addition, $CaCl_2$ and $AlCl_3$ had stimulating effect on the activity of this enzyme, but the enzyme activity was greatly inhibited by the presence of $PbCl_2$ and $HgCl_2$ (data not shown).

Substrate specificity of endo-poly(1,4-β -D-mannuronide-1,4-α -L-guluronide) lyase

Figure Ⅲ-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 (Fig. Ⅲ-5A). Interestingly, the 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 (Fig. Ⅲ-5A). Figure Ⅲ-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, my enzyme showing such specificity is a novel alginate lyase. Because the alginate lyase so far reported have proved to be either poly(1,4-β -D-mannuronide) lyase or poly(1,4-α -L-guluronide) lyase (Gacesa, 1992; Sutherland, 1995). For this connection, the author describe several possible interpretations for the substrate specificity of my enzyme.

First, it is considered to be due to the result of contamination by other alginate lyase in the purified enzyme. However, the possibility of such contamination was ruled out by both SDS-PAGE (Fig. Ⅲ-1) and IEF (Fig. Ⅲ-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.*, 1997) 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 EndoMase (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℃ for 24 h. The enzyme degraded the M-block, but not the G-block (refer to CHAPTER Ⅱ).

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, the author has purified and characterized a novel alginate lyase from *F. multivolum* K-11 that cannot be classified as EndoMase (EC 4.2.2.3) or EndoGase (EC 4.2.2.11), because the enzyme, obtained in this chapter, degraded the M-block and G-block substrates at nearly equal rates. Since such activity of alginate lyase has not been reported, the author believes that this is novel alginate lyase. Thus, the author designated the enzyme as endo-poly(1,4-β-D-mannuronide-1,4-α-L-guluronide)lyase, namely EndoMGase (Takeuchi *et al.*, 1997). The author is the first to find out the existence of EndoMGase in nature.

Table Ⅲ-1. Summary of the Purification of EndoMGase 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
Cromatofocusing	6.94	224	32.3	12.1	30.2
Ultrogel AcA-54	4.98	158	31.7	8.5	29.6

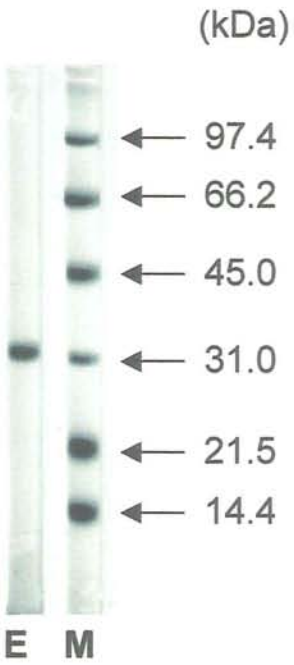


Fig. Ⅲ-1. Analysis of the Purified Enzyme by SDS-PAGE and Coomassie Brilliant Blue R-250 staining.
Lane E, purified enzyme; lane M, relative molecular mass (kDa) markers.

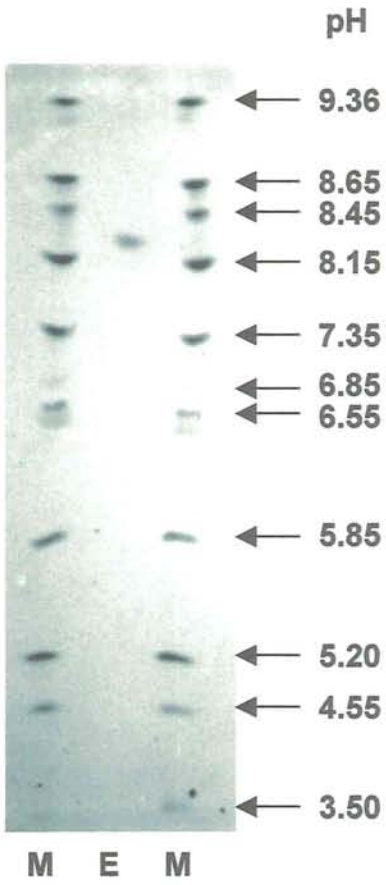


Fig. Ⅲ-2. Estimation of pI of Purified Enzyme.
Lane M, pI standard; lane E, purified enzyme.

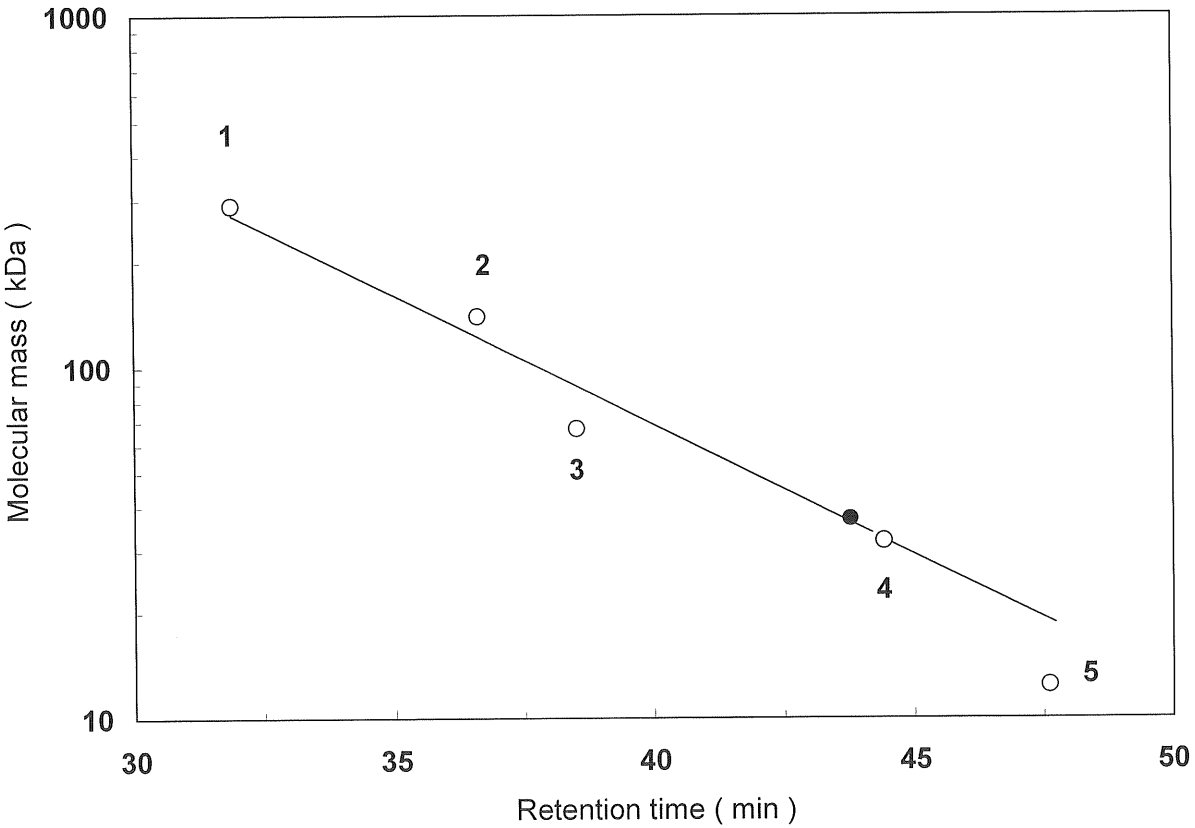


Fig. Ⅲ-3. Estimation of Molecular Mass of EndoMGase by Gel Filtration Column Chromatography.
○, molecular mass standards (Oriental Co.): 1, yeast glutamate dehydrogenase (290,000); 2, pig heart lactate dehydrogenase (140,000); 3, yeast enolase (67,000); 4, yeast adenylate kinase (32,000); 5, horse heart cytochrome C (12,400); ●, purified EndoMGase.

Table Ⅲ-2. Amino Acid Composition of EndoMGase

Amino acid	Mol percentage (%)	
	EndoMGase	EndoGase
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.
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

n.d., not determined.

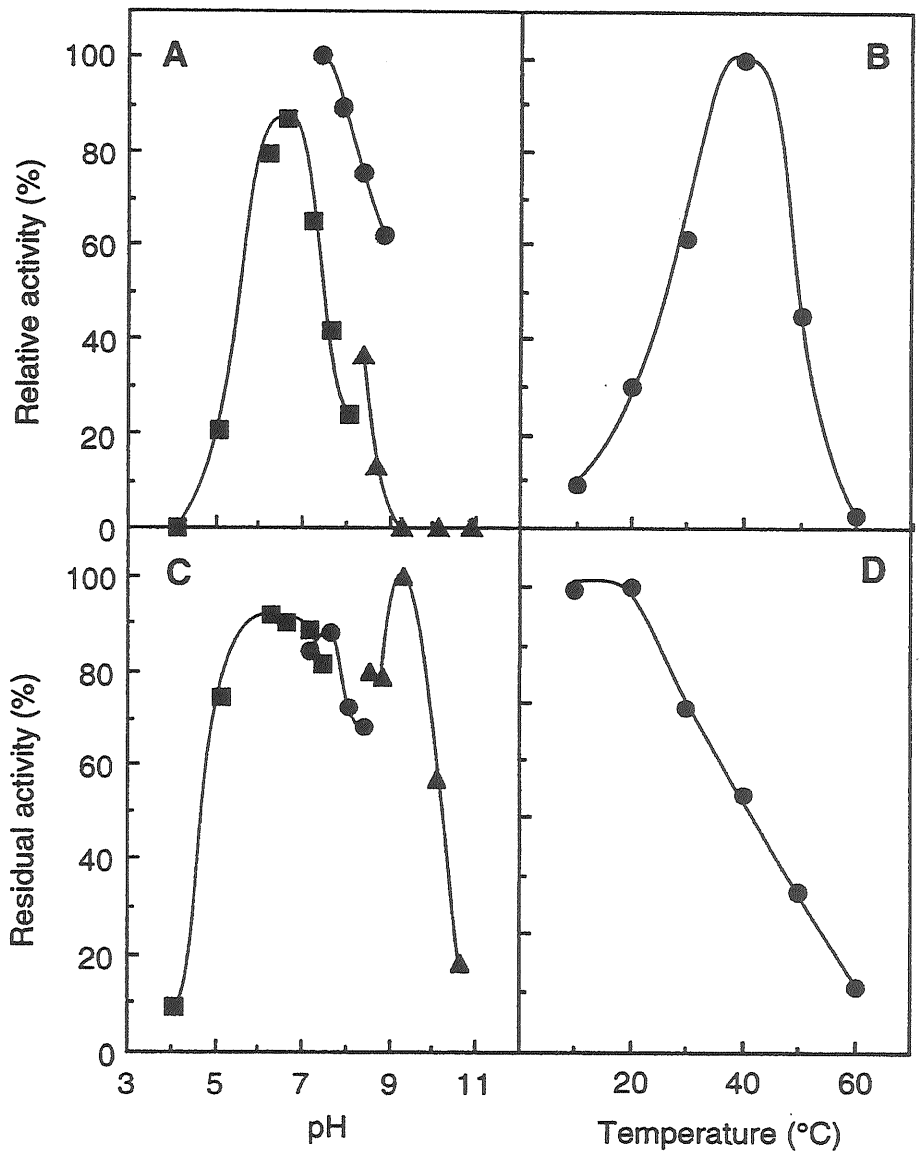


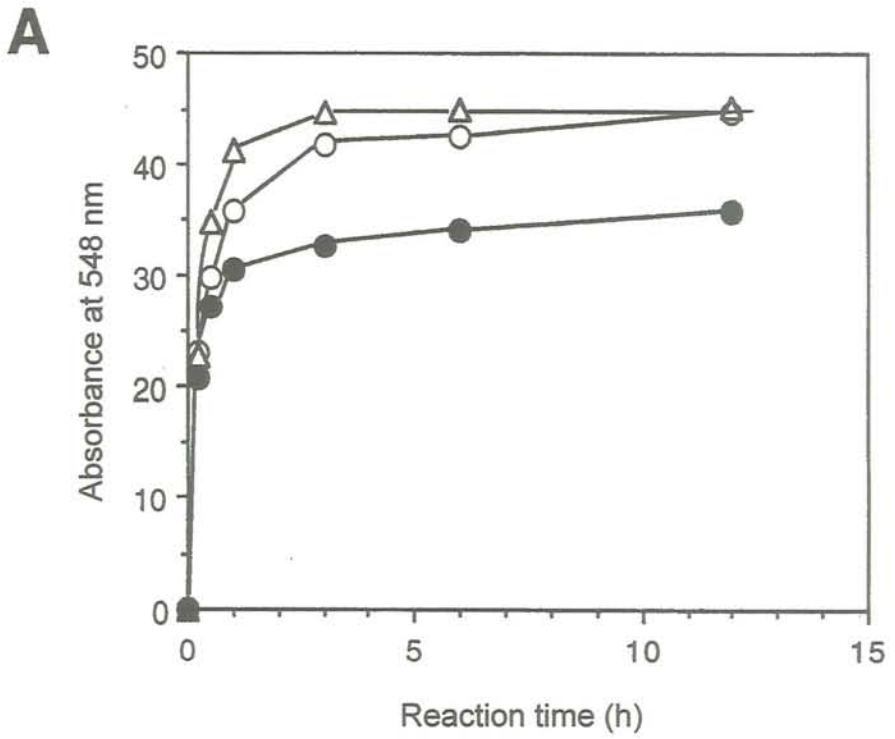
Fig. Ⅲ-4. Enzymatic Properties of Purified Enzyme.

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℃ 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. Enzyme solutions were incubated at various pH values, using McIlvaine buffer (■), Tris-HCl buffer (●), and Atkins-Pantin buffer (▲), at 25℃ 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 stability was incubated at various temperature at pH 8.0 in Tris-HCl buffer for 1 h, and residual activity was determined.

Table Ⅲ-3. Effects of Chemical Compounds on EndoMGase

Compound	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

After the enzyme solution containing a test conpounds (1×10^{-3} M) was pre-incubated in Tris-HCl buffer at pH 8.0 and 25℃ 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, ethylendiamintetraacetic acid; SDS, sodium dodecyl sulfate; PCMB, *p*-chloromercuribenzoic acid; MIA, monoiodoacetic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid.



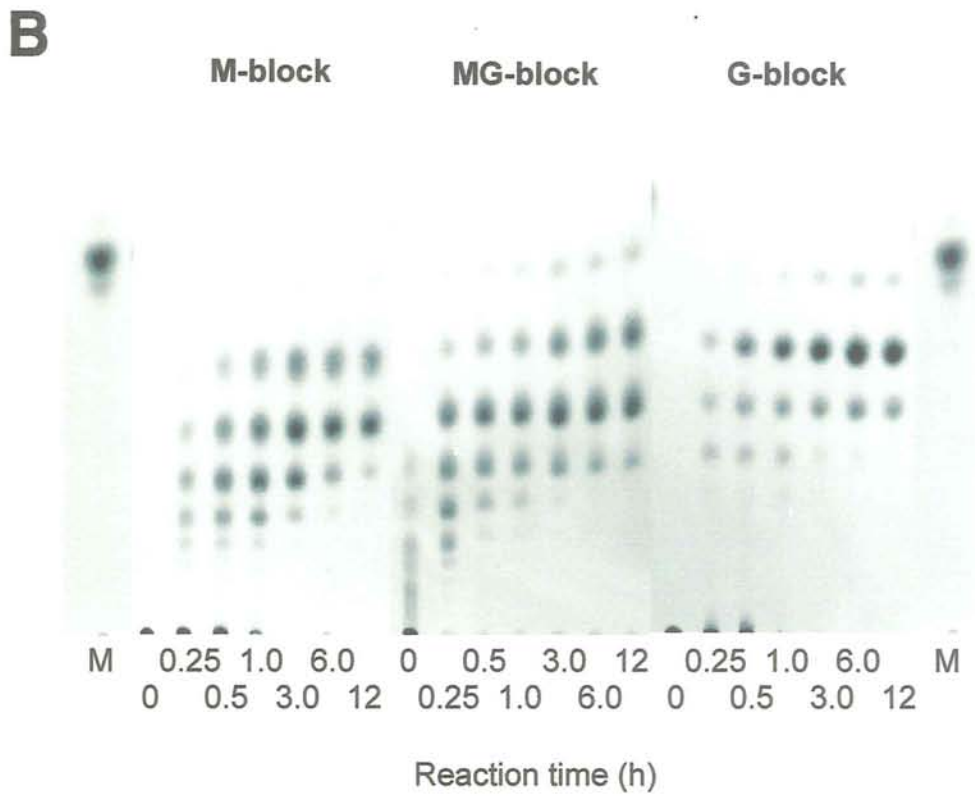


Fig. Ⅲ-5. Time-course of the Enzyme Reaction toward G-, MG- and M-blocks. A: The course of enzyme reaction followed by TBA reaction. △, G-block; ●, MG-block; ○, M-block. B; The course of enzyme reaction followed by TLC staining with sulfuric acid. Lane M, D-mannuronic acid lactone.

CHAPTER Ⅳ

A SIMPLE METHOD FOR PREPARATION OF POLY-MANNURONATE USING ENDO-POLY(1,4-α-L-GULURONIDE) LYASE

SUMMARY

A simple method for the preparation of poly-mannuronate from alginate has been developed. By making the best use of the substrate specificity of poly-guluronate lyase, the author prepared a poly mannuronate of which the properties were almost identical to those of the poly-mannuronate produced by Haug's acid hydrolysis method. This method is very useful in terms of time and labor saving.

INTRODUCTION

Alginic acid forms three block structures, namely M-block, G-block and MG-block. The development of a convenient preparation method of these three blocks is required for multiple uses of alginic acid, for example, for studying the substrate specificity of alginate-degrading enzymes. Haug *et al.*, (1967) reported a method for the preparation of the three blocks by controlled acid hydrolysis of alginate. The method has been used for about thirty years, however, the method requires a lot of time and labor because of the complication of the preparation process. For such circumstances, the author assumed that the method for the preparation of blocks is simplified by making the best use of the

specificity of alginate-degrading enzyme.

In CHAPTER Ⅰ, the author described that the enzyme (EndoGase) from *Fravobacterium multivolum* degraded both G- and MG-blocks bud did not M-block. By using EndoGase, the author assumed that M-block is still existent in the material after removal of G- and MG-blocks from alginate by the enzyme. In this connection, CHAPTER Ⅳ describes a simple method for the preparation of M-block from alginate by taking the advantage of specificity of EndoGase, and the comparison of Haug's method with my method.

MATERIALS AND METHODS

Sodium alginate

The alginate (M/G; 0.94), Duck Algin 350M-T, was the same as that described in CHAPTER Ⅰ.

Preparation of M-, G- and MG-blocks by Haug's method

The author prepared M-, G- and MG-blocks from sodium alginate according to the method of Haug *et al.* (1967). M-, G- and MG-blocks were obtained 11.2 g, 18.8 g and 13.6 g, respectively, from 100 g of the sodium alginate.

Enzyme preparation

Partially purified enzyme, prepared from the crude powder of *Flavobacterium multivolum* K-11, was used in this chapter. The enzyme was EndoGase, and was prepared as follows. A 12 g of enzyme powder (purchased from Nagase Biochemical

Co., Kyoto) was dissolved in 600 ml of 1 mM phosphate buffer (pH 6.3), and the enzyme solution was dialyzed against the buffer, followed by centrifugation to remove insoluble materials. The supernatant (650 ml, 1,833 units) was used as a crude enzyme solution. The enzyme solution degraded all of M-, G-, and MG-blocks. Then, the solution was put onto a column of CM-Toyopearl 650M (26 x 400 mm, Tohso Co., Tokyo) equilibrated with 1 mM phophate buffer (pH 6.3) to remove poly-mannuronate lyase activity. The column was washed with the same buffer at a flow rate of 2.0 ml/ min. The eluate (600 ml) containing lyase activity was concentrated to 61 ml (400 units) by ultrafiltration using an Amicon YM-3 membrane. The procedure was repeated four times to yield 1600 units of the enzyme. The combined concentrate had no EndoMase activity, but contained G- and MG-block-degrading enzyme activities. Thus, the enzyme was EndoGase, and was used for the preparation of M-block. The enzyme was stable at 4℃ for at least 6 months in 1 mM phosphate buffer containing 0.02% NaN_3, and showed optimum activities at pH 8.0 and 37℃.

Assay for alginate lyase activity

The assay was performed as described in chapter Ⅰ.

RESULTS AND DISCUSSION

Preparation of ΔM-block by enzymatic degradation

Figure Ⅳ-1 shows the flow chart for preparation of ΔM-block from sodium alginate. Sodium alginate (M/G; 0.94), 30 g, was dissolved in 1 liter of water containing 0.1 M sodium chloride, and the solution was adjusted to pH 8.0. The EndoGase

obtained above (243.5 ml, 1,600 units) was added to the substrate solution, and then more sodium alginate (70 g) was added potionwise in the course of 2 h, because of the avoidance of increasing viscosity. The resultant mixture was left for 22 h at 37°C. The reaction mixture was heated at about 100°C for 10 min to inactivate the enzyme. After cooling to room temperature, the mixture was adjusted to pH 2.85 with 1.0 N hydrochloric acid, and centrifuged to remove a small amount of insoluble material. The supernatant was adjusted to pH 1.5 with 0.1 N hydrochloric acid, and left at 4°C overnight. The resultant precipitate was collected by centrifugation, suspended in water (90 ml), and dissolved by neutralization with 1.0 M sodium carbonate. The solution (120 ml) was added dropwise into ethanol (360 ml). The resultant precipitate was washed with ethanol and ether, and dried *in vacuo* to yield ΔM-block (10.8 g).

Preparation of M-block from ΔM-block by acid hydrolysis

Figure Ⅳ-2 shows the flow chart for preparation of M-block from ΔM-block by acid hydrolysis. ΔM-block (10.8 g) prepared by the same method described above was suspended in water (90 ml) and adjusted to pH 3.0 with 0.1 N hydrochloric acid. The reaction mixture (100 ml) was heated at 100°C for 2 h to remove the Δ moiety from the ΔM-block by acid hydrolysis. The hydrolysate was neutralized with 1.0 M sodium carbonate, and the solution (130 ml) was added dropwise into ethanol (390 ml). The resultant precipitate was washed with ethanol and ether, and dried *in vacuo* to give M-block (9.7 g).

Some properties of the products and comparison with the products by Haug's method

Figure Ⅳ-3 shows the circular dichroism (CD) spectra of ΔM- and M-blocks obtained by the author method, and the CD spectrum of M-block obtained by Haug's

method. The CD spectrum of M-block from ΔM-block was almost the same as that of M-block obtained by Haug's method. The result indicates that ΔM-block was easily converted into M-block by acid hydrolysis. On the other hand, the molecular weight distribution of both M-blocks was measured by gel filtration chromatography using HPLC system described by Takeuchi *et al.* (1994). The M-block obtained by author method showed almost the same molecular distribution pattern as that of Haug's M-block (data not shown). Therefore, it is confirmed that both M-blocks were identical in physicochemical properties.

Table Ⅳ-1 shows yields, and M and G contents of the products prepared by the author and Haug's methods. M and G contents in Table 1 were measured according to the method of Morris *et al.* (1980). From the results, the yields and the contents of M-blocks were almost equal by both methods.

As mentioned above, the author could develop a new preparation procedure of Δ M- and M-blocks by the best use of the specificity of poly-guluronate lyase. The advantages of the author method are the simplification of the process, saving time required, and the reduction of the volume required for the precipitation step, as compared to the Haug's method.

The author expects that a similar procedure can be applied to prepare the G-block. Now the author is trying to find an enzyme which has M- and MG-block-degrading activities but not G-block-degrading activity.

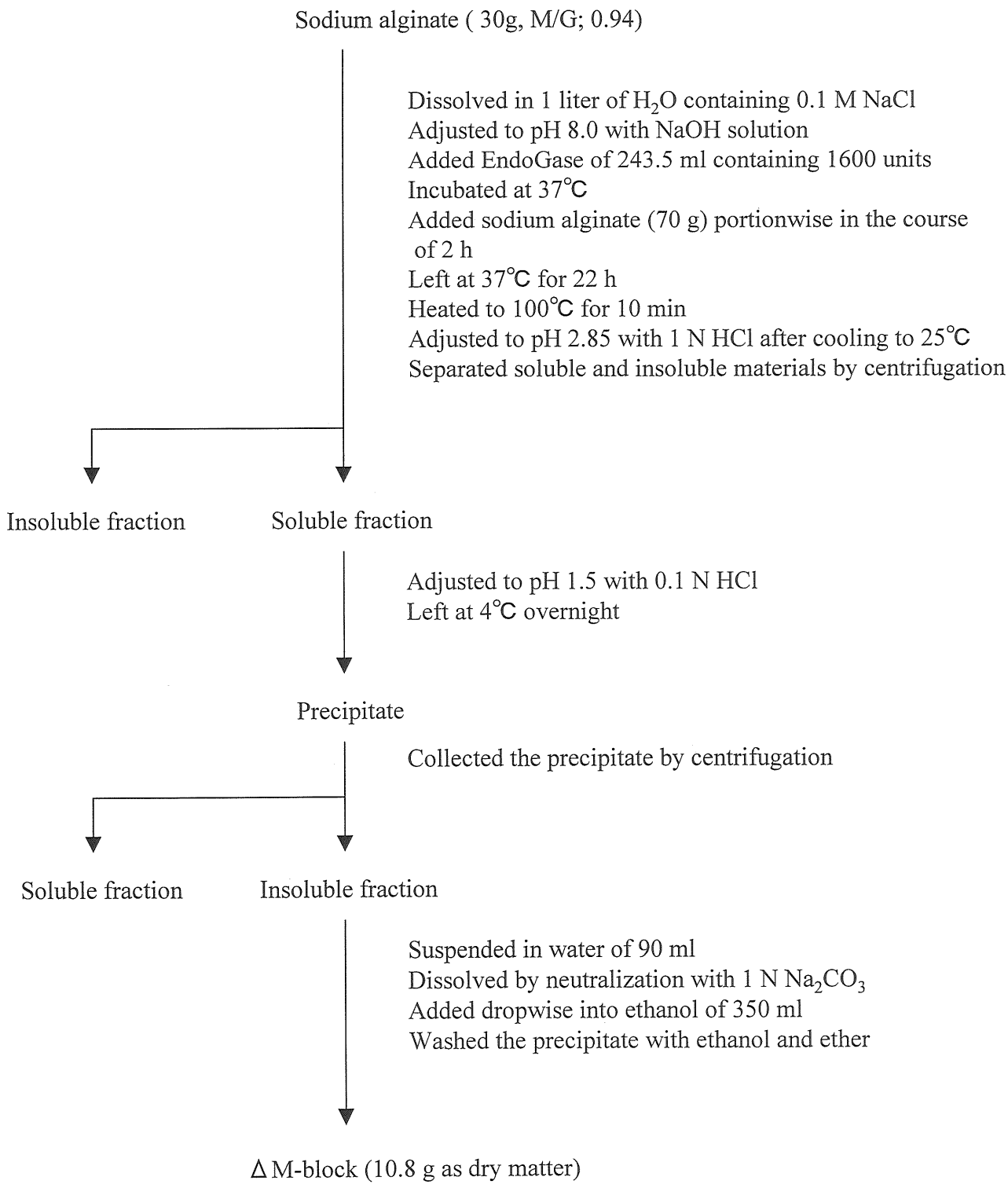


Fig. Ⅳ-1. Flow Chart for Preparation of ΔM-block from Sodium Alginate by Enzymatic Hydrolysis.

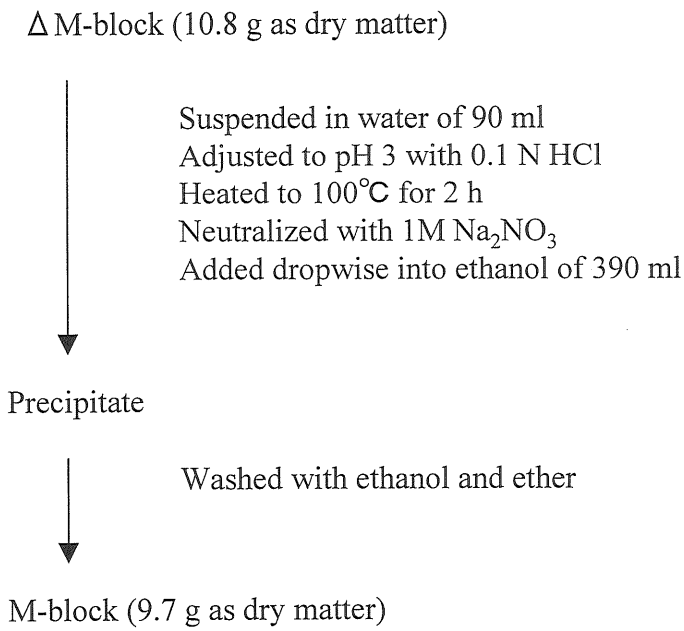


Fig. Ⅳ-2. Flow Chart for Preparation of M-block from Δ M-block by Acid Hydrolysis.

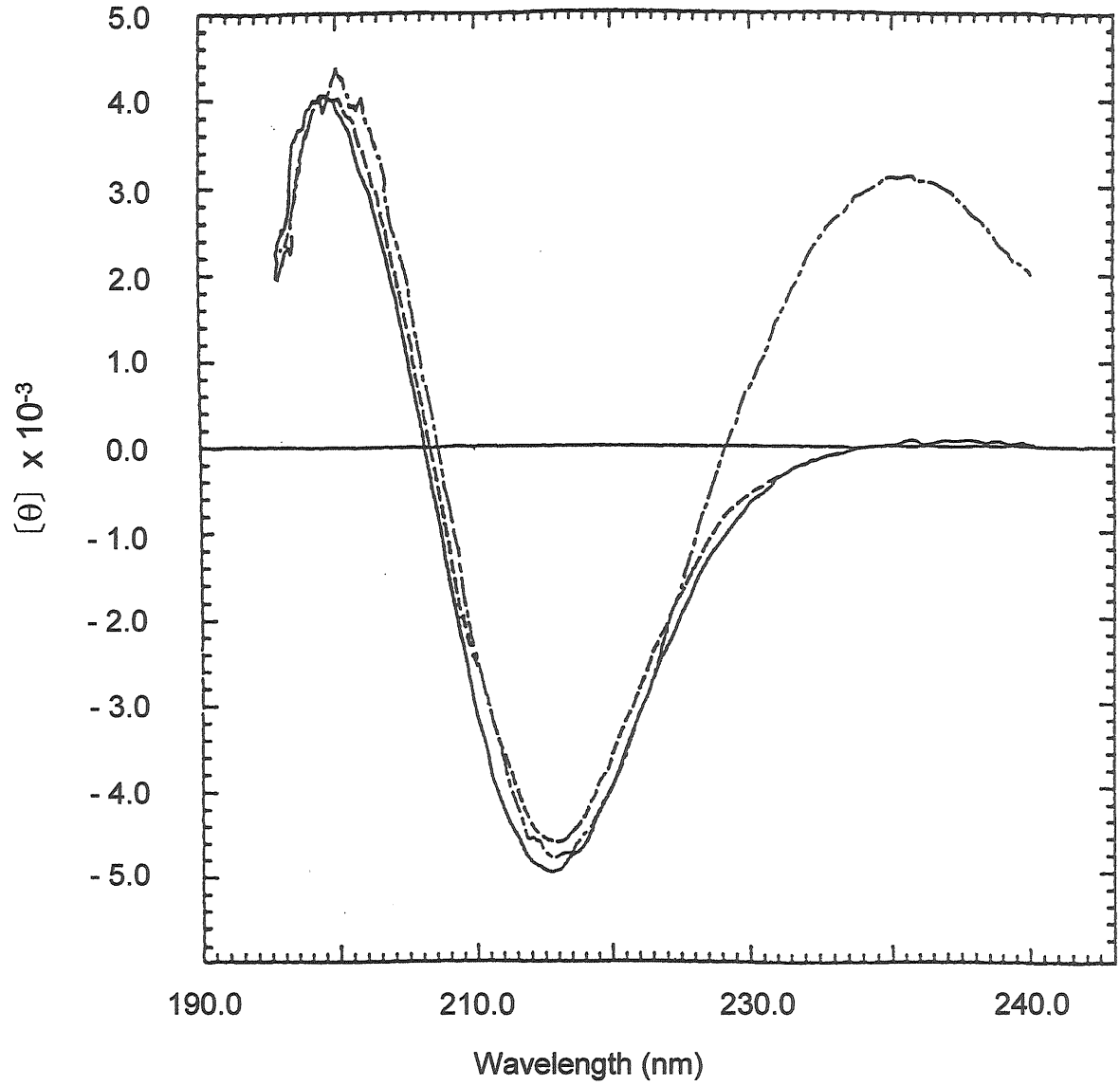


Fig. Ⅳ-3. Comparison of the Circular Dichroism Spectra of Author and Haug's M-blocks. M-block (——) and ΔM-block (—·—·—) by author method and M-block by Haug's method (------). The spectra were recorded with a 10 mm cell at pH 7 and 25℃. The sugar concentration was 1 mg /ml of each sample in water.

Table Ⅳ-1. Comparison of Yields, and M and G Contents of Products Prepared by Author and Haug's Mthods

Procedure	M-block	ΔM-block	G-block	MG-block
Author method	9.7% M% = 90.0	10.8% M% = 91.8%	Not obtained	Not obtained
Haug's method	11.2% M% = 92.2	Not obtained	18.8% G% = 78.6	13.6% M% = 58.1

The values of upper stage and lower stage in the table indicate the yield on the basis of the starting material, and M and G contents, respectively.

CHAPTER V

A METHOD FOR DEPOLYMERIZATION OF ALGINATE USING THE ENZYME SYSTEM OF *Flavobacterium multivolum*

SUMMARY

The author investigated a method for depolymerization of alginate with the crude enzyme of *Flavobacteium multivolum*. The enzyme exhibited the maximum activity at pH 7.0 and 50°C, and was stable in the pH range of 4 to 8 and at temperature up to 40°C. Enzyme activity was activated in the presence of around 0.1 M of NaCl, KCl and $MgCl_2$ in reaction mixture. The enzyme decreased the viscosities of solutions of Na-alg, K-alg and NH_4-alg, and solution of PGA. The enzyme belonged to alginate lyase and degraded Na-alg by an endo-wise manner. The time-course of change in viscosity on large scale reaction is almost the same as that of beaker scale reaction, and the clarification of alginate digest was possible by the treatment with active carbon. Actually, a 4.9 Kg of Na-alg (M/G; 1.04) was degraded with the crude enzyme (32.3 g of the enzyme powder with 31,814 units) of *F. multivolum* at pH 6.8 and 45°C for 2.5 h. The viscosity of reaction mixture, thus obtained, was about 4 centipoises (3.5%, w/w).

INTRODUCTION

In the general introduction of this thesis, the author described that alginate is

extracted from brown seaweeds, is soluble dietary fiber, and has some physiological functions. In this connection, alginate and its derivatives have been extensively used as gelling agents, emulsifiers and stabilizers in foods. However, the alginate is difficult to use for foods, especially an appropriation of alginate for beverage since the aqueous solution of alginate have high viscosity even at a concentration of 0.5%. For this reason, the author has investigated to prepare low viscous alginate solution by enzymatic method. In addition, it is great convenience from the standpoint of the users to use a crude enzyme for food processing. Thus, the author, in this chapter, dealt with some properties of crude enzyme prepared from *Flavobacterium multivolum* and the production of oligo-uronide solution from alginate with the enzyme.

MATERIALS AND METHODS

Alginates

Two kinds of sodium alginates (Na-alg), "Duck Algin" produced by Kibun Food Chemifa Co. (Tokyo), were used in this experiment. M/G ratios of the alginates were 0.79 and 0.21. The ratio was determined according to the method of Haug *et al.* (1974). Potassium alginate (K-alg) and ammonium alginate (NH_4-alg) were prepared from Na-alg with M/G of 0.79 and M/G of 0.21, respectively. The substitutional ratios of potassium ion were 85% on Na-alg of M/G; 0.79 and 88% on Na-alg of M/G; 0.21, and the ratios of ammonium ion were 63% on Na-alg of M/G; 0.79 and 59% on Na-alg of M/G; 0.21.

Propylene glycol ester of alginic acid

Two kinds of propylene grycol ester of alginic acid (PGA), "Duck loid" produced by Kibun Food Chemifa Co., were also used in this experiment. The esterificated ratios of propylene glycol were over 83% and 45%. The M/G ratios of these PGA were approximately 0.5.

Alginate-degrading enzyme

The enzyme powder, originated from *Flavobacterium multivolum* K-11, was purchased from Nagase Biochemicals (Kyoto). The enzyme powder was treated as follows; active carbon was added to culture broth to remove color materials, and then filtered for removing active carbon. The resultant solution was further filtered to remove the bacterial cells through the pore size 0.2 μ m filter, and then alcohol was added to the filtrate to precipitate the enzyme. The precipitate was lyophilized and used as the crude enzyme.

Assay for alginate-degrading enzyme activity

The activity was assayed by the following method. A 3 ml of 0.1% Na-alg (M/G; 0.79) solution dissolved in phosphate buffer (pH 6.3) was put into a test tube. The tube was preincubated for 10 min in a water bath maintained at 37℃, and then a 0.3 ml portion of enzyme solution was added to the tube. The reaction was continued for 5 min under the above condition, and the change in absorbance was measured at 235nm. One unit of enzyme activity was defined as the amounts of enzyme which caused 1.0 absorbance increment in 1 min.

Measurement of viscosity in enzyme reaction mixture

Visconic ED type (Tokyo Keiki Co. Tokyo) was used for the measurement of

viscosity. The viscosity of enzyme reaction mixture containing 1.0% of alginate was measured at 20℃.

HPLC analysis

Gel filtration high-performance liquid chromatography was carried out with a Waters 600E Multisolvent Delivery System Chromatograph (Waters Division of Millipor, Mass.) with a gel filtration column connecting Shodex OH pak KB-80M (8 x 300 mm) and KB-802.5 (8 x 300 mm) (Showadenko Co., Tokyo). Sample was applied to the columns maintained at 50℃, eluted with 50 mM $NaNO_3$ at a flow rate of 1.0 ml/ min, and the degradation products in the eluate were detected with a Waters 410 Differential Refractometer (Waters Division of Millipor, Mass.).

Thin-layer chromatography

The sugar sample after enzyme reaction was applied on TLC plate (Merck TLC plate of silicagel 60) for the characterization of degradation products from alginate and developed with a solvent system of 1-butanol : formic acid : water (4 : 6 : 1, v/v). The sugars on the plate were detected by heating at 150℃ for 5 min after spraying with concentrated sulfuric acid.

Measurement of fibrous properties

The properties were measured by the method of Prosky (AOAC, 1984).

RESULTS AND DISCUSSION

Enzymatic properties of alginate-degrading enzyme from F. multivolum K-11

Effect of temperature on enzyme activity The enzyme reaction was carried out at various temperatures (10-50℃) for 5 min at the pH 6.3 in phosphate buffer. The enzyme exhibited maximum activity at around 50℃, but almost no activity was seen at 60℃ (data not shown).

Effect of temperature on enzyme stability The enzyme solution was treated at increments of 10℃ from 10℃ to 60℃ for 15 min under pH 7.0 in McIlvaine buffer, and remaining activity was assayed. The enzyme was stable at temperatures below 40℃, but the same treatment at 50℃ showed about 60% loss of original activity. Thus, it is shown that stability of the enzyme was not sufficient.

Effect of pH on enzyme activity The enzyme reaction was carried out at various pHs (4-8 in McIlvaine buffer) and 37℃ for 5 min. The enzyme had the highest activity at around pH 7 (data not shown).

Effect of pH on enzyme stability The residual activity was assayed after exposing the enzyme solution to various pH (4-8 in McIlvaine buffer) at 25℃ for 5 h. The enzyme remained about 90-100% of the original activity after treatment in the pH range of 4.0-8.0 (data not shown).

Effects of metal compounds on enzyme reaction

Effects of NaCl, KCl and $MgCl_2$, which have been used in food industry, were tested for alginate-degrading enzyme. The reaction mixture was composed of 3 ml of 0.1% Na-alg (M/G; 0.79) solution (pH 6.8) containing a range of 0 to 1.0 M of the salts and 0.3 ml of enzyme solution (0.57 units). Then, the reaction was carried out at 37℃ for 5 min, and the amount of degradation product was determined with increase of

absorbance at 235 nm. Figure V-1 shows the effect of salt concentration on enzymatic degradation of alginate. The degradation was accelerated (about 4- to 7-fold) under the existence of around 0.1 M of the salts, especially under 0.1 M of $MgCl_2$. However, it is not clear from this experiment whether $MgCl_2$ acted to aid the enzymatic degradation of alginate, or acted as an activator for the enzyme.

Time-course of change in viscosity and absorbance at 235 nm by enzyme reaction in the presence of NaCl or $MgCl_2$

The reaction mixture was composed of 100 ml of 1% Na-alg (M/G; 0.79) solution (pH 6.5) containing 0.05 M of salt (NaCl or $MgCl_2$) and 10 ml of enzyme solution (4.75 units). The enzyme reaction was continued at 37°C for 40 min, then the viscosity and the absorbance at 235 nm of the reaction mixture were determined at intervals of 20 min. Figures V-2A and V-2B show the time-courses of decrease of the viscosity and increase of the absorbance, respectively. The viscosity of reaction mixture without salt reduced to 4 centipoises at 120 min, but the mixture containing 0.05 M NaCl or $MgCl_2$ reached the same viscosity at 75 min (Fig. V-2A). On the other hand, the absorbance at 235 nm of the reaction mixture increased linearly with the elapse of reaction time (Fig. V-2B). Thus, the result indicates that alginate was decomposed by lyase reaction. Because the enzyme produced 4,5-unsaturated products, which had absorbance at 235 nm, from alginate, and absorbance increased as the reaction time proceeded.

Time-course of enzymatic degradation of various alginates

A 10 ml of enzyme solution containing 4.28 units in 0.1 M phosphate buffer (pH 6.8) was added to each 100 ml of 1.0 % Na-alg, K-alg, NH_4-alg, or PGA dissolved in

water. The enzyme raction was continued at 37°C for 120 min, and then viscosity, absorbance at 235 nm and pH of the reaction mixture were determined at 30, 60, 90 and 120 min. Figure V-3 shows the time-course of changes in viscosity, absorbance at 235 nm and pH during enzyme reaction. The enzyme decreased the viscosity of alginate solutions and increased the absorbance at 235 nm as reaction time proceeded (Figs. V-3A and V-3B). The results indicate that the crude enzyme decomposed the various alginates, namely alginate having different M/G ratios, and NH_4-, Na-, K-algs etc., at almost the same speed. On the other hand, the decrease of viscosity and the increase of absorbance at 235 nm, in the case of PGA, were similar to other alginates at the beginning (within 30 min) of the enzyme reaction, whereas the reaction rate leveled off thereafter (Figs. V-3A and V-3B). This suggests that glycol ester was saponified to Na-alg and glycol at the initial pH 6.5 of the reaction, and the pH of reaction mixture fell to 4.0 or 5.0 within 30 min by incorporating cation (Na^+) to alginic acid (Fig. V-3C). That is to say, the activity of this enzyme markedly fell at lower pH range such as pH 5 or 4 that the relative activity for optimal pH was less than 20%. In addition, it became clear that the glycol ester of alginic acid was saponified at pH higher than 5. Therefore, the glycol ester is used for sour beverage containing citric acid, lactic acid, etc.

Change in molecular weight of sodium alginate during enzyme reaction

Enzyme powder (26.4 mg, total activity; 26.0 units) was added to 400 ml of 1.0% Na-alg (M/G; 0.79) dissolved in water, and the reaction was continued at pH 6.8 and 37°C. Further 13.2 mg (total activity; 13.0 units) and 9.9 mg (total activity; 9.75 units) of the same powder were added to the reaction mixture after 20 h and 44 h of enzyme reaction, respectively. At certain intervals of time (1, 2, 3, 20, 44 and 85 h), 50 ml of the

mixture was taken out to a 200-ml flask and heated immediately to about 100℃ for 5 min to inactive the enzyme. Active carbon powder (2.5 g, Takeda Yakuhin Co., Osaka) and celite (0.83 g, Tokyo Pearlite Kogyo Co., Tokyo) were added to the flask, and the sample was stirred to adsorb protein and color compounds. After filtration with a glass filter precoated with celite to remove solid materials, the resultant filtrate was lyophilized. The sample thus obtained was dissolved in 50 mM $NaNO_3$ solution so as to adjust the sugar concentration to 0.1%.

For determining the change in the molecular weight distribution, the sample was subjected to HPLC. The molecular weight was determined by using pullulan (Showdex standard P-82, Showadenko Co., Tokyo) and glucose as standard samples. The sugars eluted from the column were detected by a differential refractometer (Waters 410, Waters Division of Millipor, Mass.). Figure Ⅴ-4 shows the change in molecular weight of Na-alg during enzyme reaction. The peak gradually shifted to right side (low molecular weight size) with one peak during 3 h of the reaction. On the other hand, the viscosity of the reaction mixture was 4.05 centipoises, and the dietary fiber contents was 66.0% (data not shown). Thereafter, the reaction proceeded to show the chromatogram with shoulder. The result indicated that Na-alg was degraded endo-wise by the crude enzyme. The lyophilized sample obtained above, on the other hand, was dissolved in deionized water to a concentration of 1.0%, and 5 μl of the sample was applied on TLC. Figure Ⅴ-5 shows TLC of the time-course of enzymatic degradation of Na-alg. The TLC demonstrated that several kinds of oligo-uronide appeared from 20 h of reaction and did not disappear even after 85 h, and mono-uronide was not detected during the entire course of enzyme reaction. Thus, the result indicated that the crude enzyme did not contain an exo-type lyase.

Large scale preparation of sodium alginate digest

Actually, Na-alg (M/G; 1.04) was degraded with the crude enzyme according to the procedure described above. The large scale preparation of Na-alg digest was done as follows. A 4.9 kg of Na-alg was dissolved in 135 *l* of water in a stainless steel vessel, and the solution was adjusted to pH 6.8 and 45℃ with slowly stirring (20–30 rpm). Enzyme powder (32.3 g including 31,811 units) was added to the solution in the vessel. Throughout the reaction, the pH of reaction mixture was maintained at 6.8 by the addition of NaOH or HCl solution. As a control experiment (small scale), a 14.5 g of Na-alg (M/G; 1.04) was dissolved in 400 ml of water in a 500-ml beaker at 45℃, and enzyme powder (95.7 mg including 94.3 units) was added. The course of change in viscosity was determined at interval of 30 min. The time-course of decrease of viscosity on the large scale reaction was almost equal to control. After 2.5 h from beginning of reaction, the viscosity of reaction mixture was about 4 centipoise. The reaction mixture after 2.5 h from the beginning of reaction was treated as follows. Active carbon powder (1,260 g) was added to the mixture for the clarification of solution, followed by filtration with a filter press precoated with cellulose powder (1,035 g) and kieselguhr (2,340 g).

In the present experiments, the author made studies on preparation of low viscosity alginate with dietary fibrous property by enzymatic methods. In the large scale preparation of Na-alg digest, the author succeeded to obtain low viscosity alginate with dietary fibrous property. Therefore, the Na-alg at 2.5 h reaction may be usable as a material for food and beverage containing dietary fiber or a material supporting the growth of Bifidobacteria in the human intestinal tracts (Akiyama *et al.*, 1992). On the other hand, the crude enzyme of *F. multivolum* K-11 accumulated several kinds of

origo-uronides after the middle stage (20h) of reaction (Fig. Ⅴ-5). Such oligo-uronides may also be usable as a material promoting the growth of some plants (Matsume *et al.*, 1994; Tomita *et al.*, 1994; Yonemoto *et al.*, 1993), namely as an elicitor or an oligosaccharine, because it is known to have the above function.

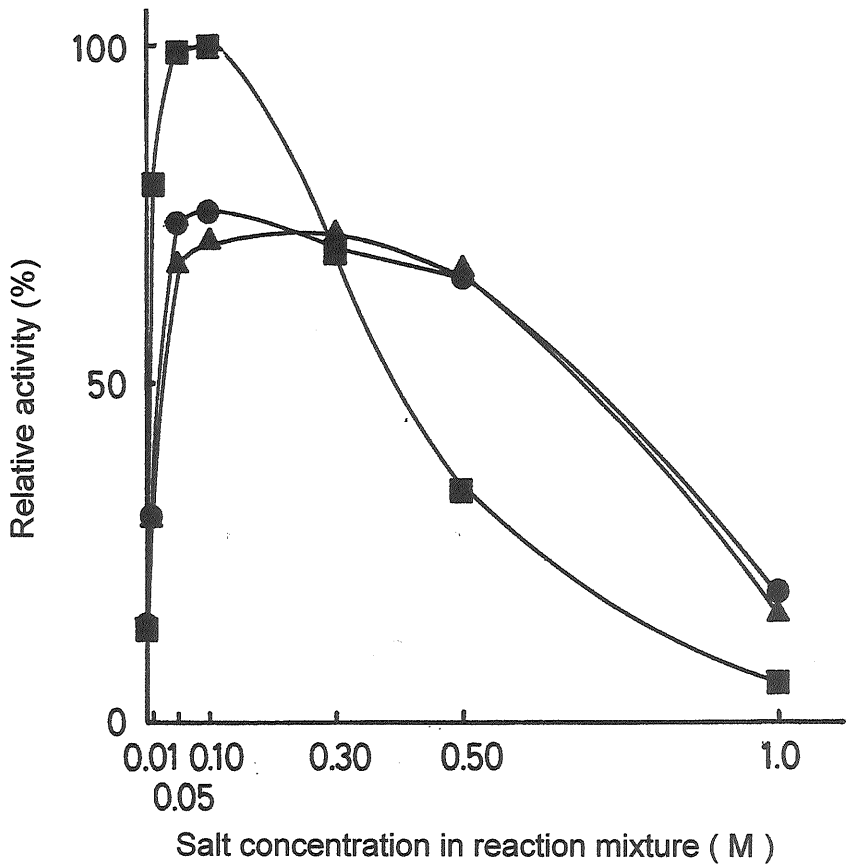


Fig. V-1. Effect of Salt Concentration on Enzyme Reaction.
- ●-, (NaCl); - ▲-, (KCl); - ■-, ($MgCl_2$)

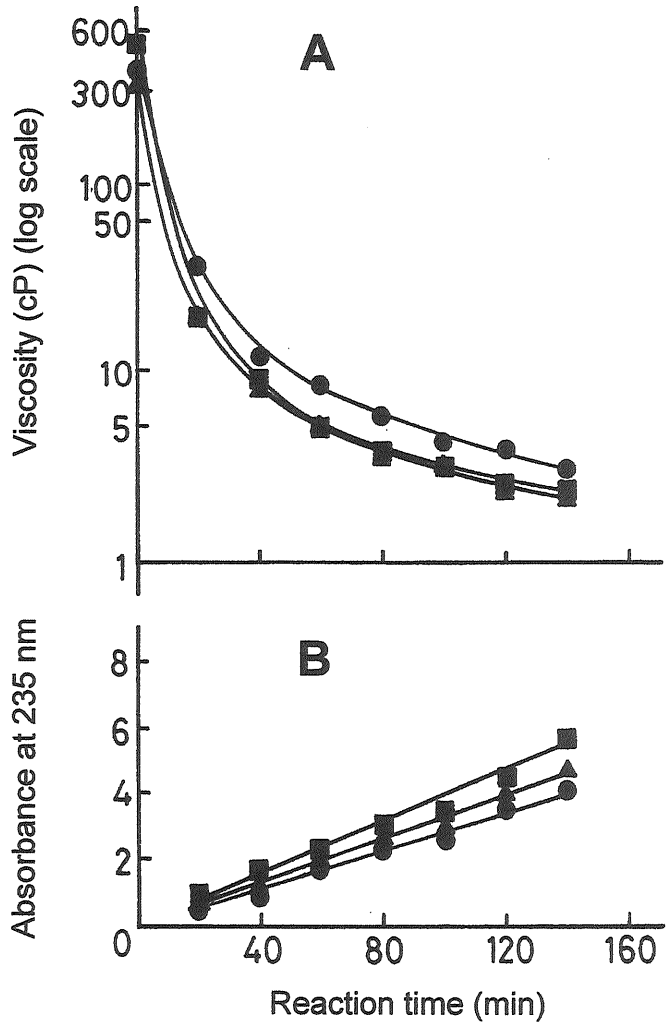


Fig. V-2. Time-course of Change in Viscosity and Absorbance at 235 nm by Enzyme Reaction in Presence of NaCl or $MgCl_2$.
A, change of viscosity; B, change of absorbance at 235 nm. -●-, (control, without salt); -▲-, (0.05 M NaCl); -■-, (0.05 M $MgCl_2$).

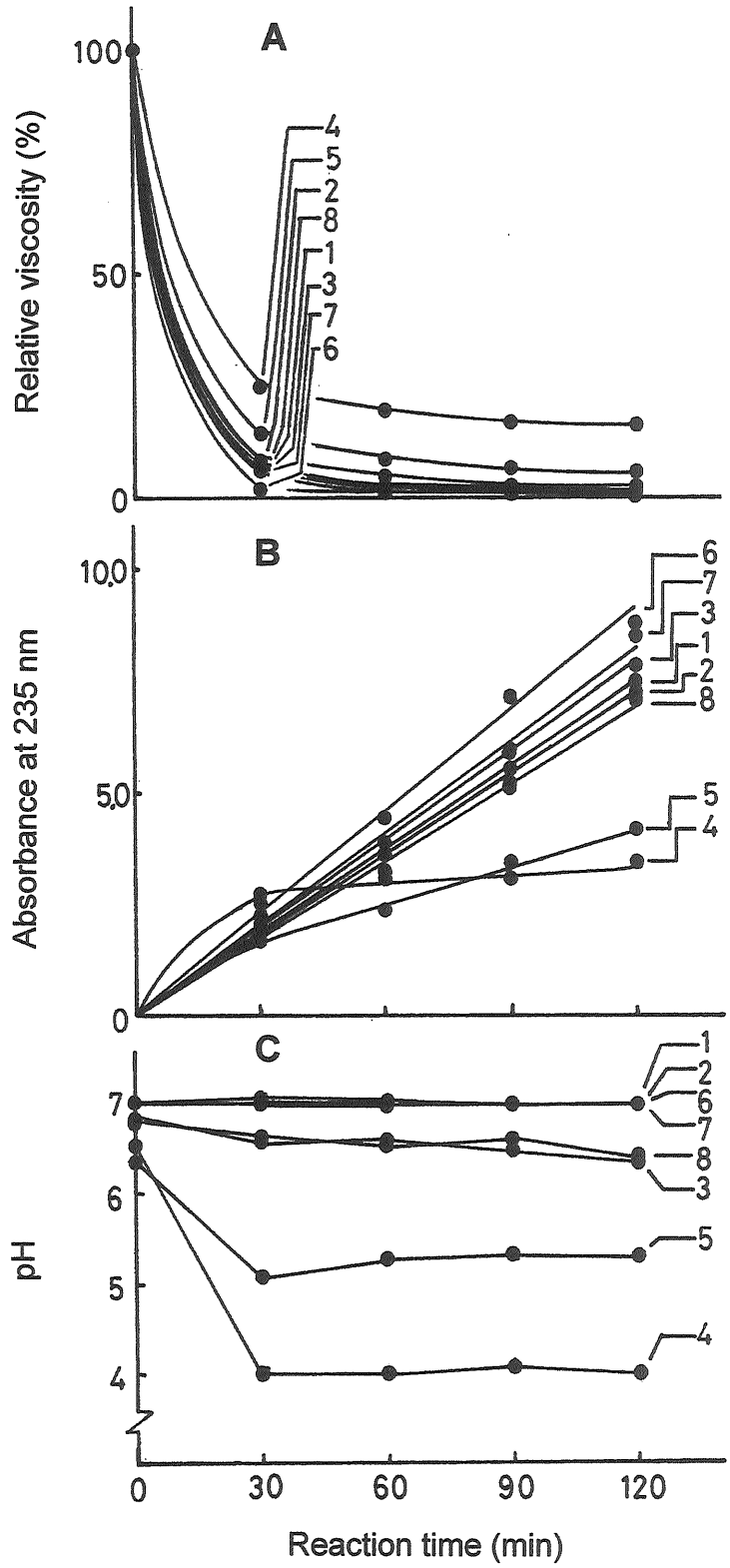


Fig. V-3. Change of Enzymatic Degradation of Various Alginates.
A, change of viscosity ; B, change of absorbance at 235 nm; C, change of pH.
1 (sodium alginate, M/G; 0.79), 2 (potassium alginate, M/G; 0.79), 3 (ammonium alginate, M/G; 0.79), 4 (PGA, esterized ratio; 83%), 5 (PGA esterized ratio; 45%), 6 (sodium alginate, M/G; 0.21), 7 (potassium alginate, M/G; 0.21), 8 (ammonium alginate, M/G; 0.21).

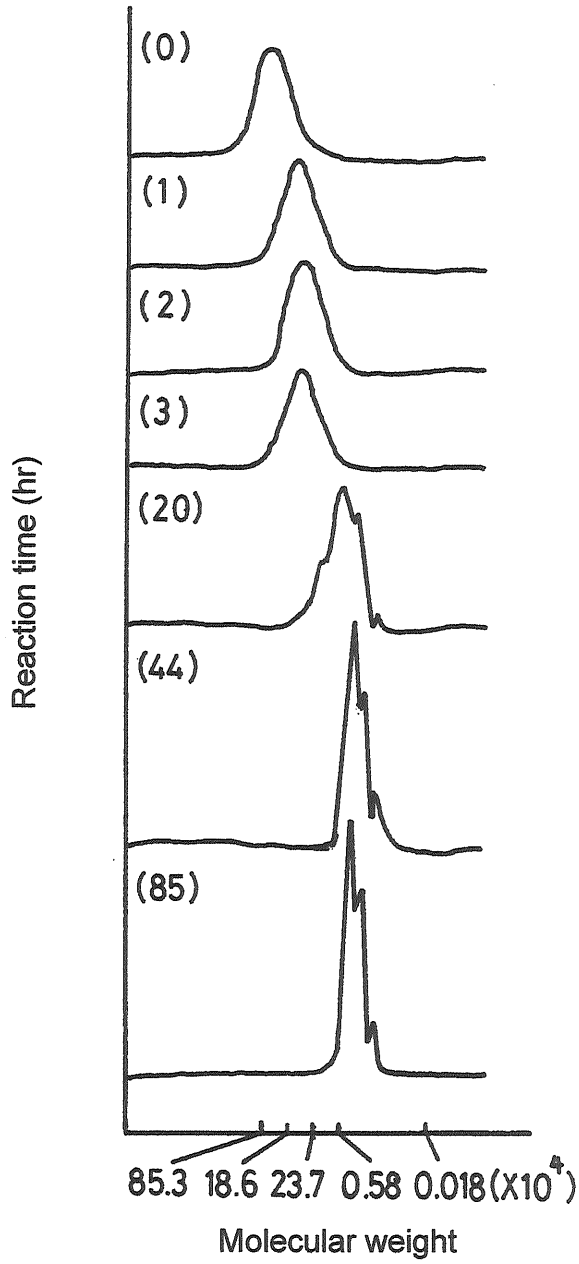


Fig. V-4. Change of Molecular Weight of Na-alginate during Enzyme Reaction.

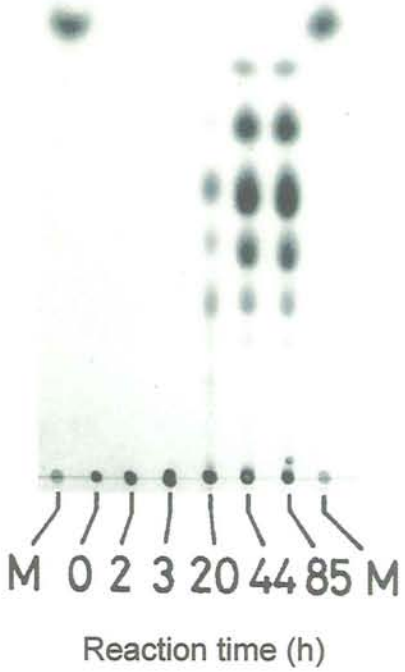


Fig. V-5. TLC of Course of Enzymatic Degradation of Na-alginate. M, authentic D-mannuronic acid lactone.

GENERAL CONCLUSION

The author attempted to find out alginate-degrading enzyme, and obtained Multivolum enzyme and Abalone Acetone Powder as enzyme sources. From the results of purification of their enzymes and characterization of the purified enzymes, it became clear that the Multivolum enzyme included both of EndoGase and EndoMGase, and Abalone Acetone Powder included EndoMase.

A summary of well-defined alginate-degrading enzymes (alginate lyases), though difficult to list up all enzymes relating to the lyase, is shown in Table GC-1. The number of alginate lyase was a few, and EndoMGase has not been published when the author started this study. However, the number increased after that, and Iwamoto *et al.* (2001) recently reported that *Altermonas* sp. produced a bifunctional alginate lyase, namely EndoMGase.

In CHAPTER Ⅰ, the author obtained an alginate lyasse from Multivolum enzyme produced by *Flavobacterium multivolum* K-11. The lyase was classified as an EndoGase [endo-poly (1,4-α-L-guluronide) lyase, EC 4.2.2.11] because the enzyme acted on G- and MG-blocks but did not on M-block.

In CHAPTER Ⅱ, a purified alginate lyase was obtained from Abalone Acetone Powder. The enzyme degraded M-block more than MG-block but did not act on G-block. Furthermore, the enzyme produced various DPs of oligouronic acid having unsaturated sugar from M-block. Thus, the enzyme was classified as an EndoMase [endo-poly (1,4-β-D-mannuronide) lyase, EC 4, 2, 2, 3].

In CHAPTER Ⅲ, the author obtained an alginate lyase purified from Multivolum enzyme. The lyase decomposed both of G- and M-blocks at nearly equal rate, and also MG-block. Moreover, the enzyme produced several kinds of unsaturated oligomers

from G-, M- and MG-blocks. The alginate lyase showing such the substrate specificity has not been discovered at that time. Thus, the author classified the enzyme into a novel alginate lyase, namely an EndoMGase [endo-poly (1,4-β-D-mannuronide-1,4-α-L-guluronide) lyase].

The characteristics of above three enzymes, namely EndoGase, EndoMase and EndoMGase obtained by the author, are summarized in Table GC-2. In general, many of alginate lyase so far known have some similar properties, that is, optimum pH for enzyme activity was present in the alkali side (pH7～9) except that the pH of *Dendryphiella salina* (Shimokawa *et al.*, 1997) was 5, pH stability had a wide range of pH4～10, optimum temperature for the activity was low (around 40℃) except for 70℃ of EndoGase from *Streptomayces violaceoruber* (Shimokawa, 1997). On the other hand, molecular masses of many alginate lyases were present in the range of about 30-kDa to 50-kDa, and pIs of the enzymes were present in alkali side except for pI 3.65 fo EndoGase from *D. salina* (Shimokawa *et al.*, 1997). There are two kinds of alginate lyases on the sensitivity of EDTA. In this case, EndoGase was sensitive to EDTA but both of EndoMase and EndoMGase were no sensitive. Moreover, the enzyme activities of three lyases were decreased by the addition of MIA, NBS, etc. This is suggested that amino acid residues containing SH-group and triptophan residue of the enzymes participated in enzyme activity. This result was also observed in alginate lyases from *E. cloacae* (Nibu *et al.*, 1995) , *D. salina* (Shimokawa *et al.*, 1997) and mid-gut gland of Turbo cornutus (Muramatsu and Egawa, 1982). Most of alginate lyases have required some metal ions for maximal activity (Gacesa, 1992). Three enzymes obtained by the author were also activated by the presence of metal ion such as Mn^{2+} and Ca^{2+}. Finally, the DP of end products from alginate reached within the range of 2 to 4 or 4 to 5 as well as other alginate lyases.

From above general properties, three alginate lyases in CHAPTERs Ⅰ, Ⅱ and Ⅲ resembled each other on enzymatic and phisico-chemical properties, and the properties also resembled the properties of other alginate lyase so far known.

In CHAPTER Ⅳ, a simple method for the enzyme preparation of ΔM-block from alginate has been developed by making the application of the substrate specificity of EndoGase from Multivolum enzyme. This method was advantageous for the preparation of ΔM-block, and the ΔM-block was easily converted into M-block by acid hydrolysis. Furthermore, the advantages of this method were the simplification of the process, saving time required, the reduction of the volume required for the precipitation step, and so on.

In CHAPTER Ⅴ, the author investigated the depolymerization method of alginate by Multivolum enzyme to obtain the basic data and depolymerization products for a feasibility study of industrialization. The enzyme activity was remarkably activated in the presence of around 0.1M of NaCl, KCl or $MgCl_2$ in reaction mixture. Moreover, the enzyme decreased the viscosities of solutions of Na-alg, K-alg, NH_4-alg and PGA. Actually, a 4.9 kg of Na-alg was depolymerized with 32.3g of Multivolum enzyme at pH6.8 and 45℃ for 2.5h. The viscosity of the reaction mixture fell to 4 centipoises at the concentration of 3.5 % (w/w), and the mixture contained various DPs of oligouronic acids having 4,5-unsaturated sugar.

A depolymerization method of alginate by enzyme has been required for the utilization of alginate oligosaccharides. However, no report was found concerning the method until the author started the study of alginate lyase. Recently, Matsubara *et al.* (1998) proposed the degradation study of sodium alginate in a continuous stirred tank reactor using the alginate lyase of *Flavobacterium* sp. immobilized with chitosan beads. The degradation products were composed of oligomers of DPs from di- to hexa-

saccharides which were the same oligomers as the author showed in Fig. V -5. Multivolum enzyme originated from *Flavobacterium multivolum* K-11 was suitable for the depolymerization of alginate as degraded three blocks of alginate nearly equally.

The author described in GENERAL INTRODUCTION that alginates and their derivatives are widely used in various fields, and acidic oligosaccharaides deriving from alginate show physiological activities on some plants and microoganisims. Moreover, Oku *et al.* (1998) and Hiura *et al.* (2001) recently reported the effects of partially hydrolyzed alginate on defecation and fecal conditions, and antihypertensive effects of alginate oligosaccharides, respectively. The studies on the alginate lyase and utilization of alginate-degrading products have recently become an interesting subject. The author anticipate a practical application of this thesis for further utilization of marine biomass.

Table GC-1. The Type Classification of Alginate Lyases Originated from Various Enzyme Sources

Type of alginate lyase	Enzyme source	Reference
EndoGase (EC4.2.2.11)	*Flavobacterium multivolum* (microorganism)	The author *et al.*, 1997
	Enterobacter cloacae (microorganism)	Nibu *et al.*, 1995
	Streptmyces violaceoruber (microorganism)	Shimokawa, 1997
	Klebsiella aerogenes (microorganism)	Lange *et al.*, 1989
	Vibrio harvei (microorganism)	Kitamikado *et al.*, 1992
	Corynebacterium sp. (microorganism)	Matsubara *et al.*, 1998
	Pseudomonas alginovora (microorganism)	Boyen *et al.*, 1990
EndoMase (EC4.2.2.3)	Abalone Acetone Powder (Marine molluscs)	This thesis, 2001
	Turbo corunutus (Marine molluscs)	Muramatsu *et al.*, 1980
	Littorina sp. (Marine molluscs)	Lyudmila *et al.*, 1974
	Vibrio alginolyticus (microorganism)	Kitamikado et al., 1992
	Bacillus circulans (microorganism)	Hansen *et al.*, 1984
	Pseudomonas aeruginosa (microorganism)	Linker *et al.*, 1984
	Dendryphiella salina (microorganism)	Shimokawa *et a*l., 1997
EndoMGase	*Flavobacterium multivolum* (microorganism)	The author *et al.*, 1997
	Altermonas sp. (microorganism)	Sawabe *et al.*, 1997, Iwamoto *et al.*, 2001

EndoGase, endo-poly(1,4-α-L-guluronide) lyase;
EndoMase, endo-poly(1,4-β-D-mannuronide) lyase;
EndoMGase, endo-poly(1,4-D-mannuronide-1,4-α-L-guluronide) lyase.

Table GC-2. Comparison of Characteristics of Three Alginate Lyases Obtained by the Author

Type of lyase	EndoGase	EndoMase	EndoMGase
Origin	*Flabobacterium multivolum*	Abalone Acetone Powder	*Flabobacterium multivolum*
pH optimum	8	7.5 – 9	7.5
pH stability	6 – 9	4 – 8	6 – 9
Temp. optimum	40 ℃	30 ℃	40 ℃
Thermal stability	< 30 ℃	< 30 ℃	< 20 ℃
Molecular mass	43 k-Da	32 k-Da	32 k-Da
pI	8.7	—	8.2
Effect of chemical compound	Inhibited by EDTA, PCMB, MIA TNBS, NBS	Inhibited by PCMB, MIA NEM, TNBS NBS	Inhibited by SDS, MIA, NBS
Effect of metal ion	Activated by Mn^{2+}, Ca^{2+}, Fe^{2+}	Activated by Mn^{2+}, Ca^{2+}, Mg^{2+}	Activated by Ca^{2+}, Al^{3+}
	Inhibited by Cd^{2+}, Hg^{2+}, Ba^{2+}, Co^{2+}	Inhibited by Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+}	Inhibited by Pb^{2+}, Hg^{2+}
Degradable substrate	G-block MG-block	M-block MG-block	G-block M-block MG-block
Main products (DP)	2-4 from G-block 2-7 from MG-block	2-4 from M-block 4-7 from MG-block	2 and 3 from G-block 2-4 from M-block 2-4 from MG-block

—; not determined.

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