

## Characterization of Recombinant PolyG-Specific Lyase from a Marine Bacterium, *Streptomyces* sp. M3

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A new alginate lyase gene of marine bacterium *Streptomyces* sp. M3 had been previously cloned in pColdI vector and transformed into *E. coli* BL21 (DE3). In this study, M3 lyase protein without signal peptide was overexpressed by induction with IPTG and purified with Ni-Sepharose affinity chromatography. The absorbance at 235 nm of the reaction mixture and TLC analysis showed that M3 alginate lyase was a polyG-specific lyase. When M3 lyase was assayed with substrate for 10 min, optimum pH and optimum temperature were pH 9 and 60°C. For the effect of 1mM metal ion on M3 lyase activity, Ca<sup>++</sup> and Mn<sup>++</sup> ions increased the alginate degrading activity by two-fold, whereas Hg<sup>++</sup> and Zn<sup>++</sup> ions inhibited the lyase activity completely. Mg<sup>++</sup>, Co<sup>++</sup>, Na<sup>+</sup>, K<sup>+</sup>, and Ba<sup>++</sup> did not show any strong effects on alginate lyase activity.

**Key words** : Alginate lyase, polyG-specific lyase, recombinant, overexpression

### Introduction

Alginic acid is a linear copolymer of  $\alpha$ -L-guluronate (G) and its C5 epimer,  $\beta$ -D-mannuronate (M). This acidic polysaccharide is a cell wall component of brown seaweeds like *Laminaria* and *Undaria* [10,11]. Some bacteria including to genera *Azotobacter* and *Pseudomonas* also produce alginate with O-acetylated form on C2 or C3 position, which can provide the roles of colonization on food surface and protection from toxic environment [6,18]. Sodium alginate extracted from brown algae is widely used in food industry and biotechnology [7,20], whereas bacterial alginate is useful for the production of micro- or nanostructures suitable for medical applications [8,19].

Macromolecule alginate can be depolymerized by alginate lyase through  $\beta$ -elimination reaction and degraded to one saturated and one unsaturated oligouronate [25]. Recently, alginate lyase has been used to analyze alginate fine structure for understanding its physico-chemical properties and to obtain the protoplasts of seaweeds for the use in the food industry and for manufacturing of other industrial materials [3-5,12]. And also, alginate lyase has been studied for the production of the functional oligoalginates [1,13] and the cure of cystic fibrosis patient who are suffered by mucoid *Pseudomonas aeruginosa* and the control

of biofilm formation of bacteria [2,9]. Alginate lyases belonging to polysaccharide lyase group is classified to poly-mannuronate lyase (EC 4.2.2.3) and poly-guluronate lyase (EC 4.2.2.11) according to substrate specificities [22,23].

In previous study, alginate degrading marine bacterium was isolated from brown seaweed and identified to *Streptomyces* sp. designated to strain M3 [14]. PolyG-specific alginate lyase gene from strain M3 was cloned and overexpressed in *E. coli* BL21 (DE3). In this study, overexpressed alginate lyase was purified and biochemical properties were characterized.

### Materials and Methods

#### Cloning and overexpression of M3 alginate lyase

Protein sequence of alginate lyase from *Streptomyces* sp. M3 was deduced from the nucleotide sequence and registered as FJ644691 and ACN56743 in GenBank, respectively. The signal peptide sequence of cloned alginate lyase was predicted by using SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>). The secreted protein without signal peptide was cloned to expression vector, pColdI according to previous study [14]. Briefly, recombinant *E. coli* containing pColdI/M3 lyase gene was cultured in LB media with 50  $\mu$ g/ml ampicillin and cloned alginate lyase protein was overexpressed by induction with IPTG at 15°C. To analyze the overexpressed proteins, the proteins of cultured recombinant were sepa-

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rated on two 12% SDS-PAGE gels. The proteins on one gel were stained with Coomassie Blue R-250 and proteins on another gel were transferred to nitrocellulose paper for immunoblotting analysis.

#### Purification of M3 alginate lyase by affinity chromatography

Overexpressed M3 alginate lyase was purified for characterization by using Ni-Sepharose affinity chromatography according to Kim *et al* [15]. Cell pellets were sonicated in 50 mM potassium phosphate buffer (pH 7.2) containing 20 mM imidazole, 0.5M NaCl, 1 mM PMSF using ultrasonicator Vibra Cell VCX400 (Sonics & Materials Inc, USA). Overexpressed (His)<sub>6</sub>-tagged fusion protein was eluted with 50 mM phosphate buffer containing 300 mM imidazole and 0.5 M NaCl. Imidazole in eluted protein fraction was removed using HiTrap<sup>TM</sup> desalting column (Amersham Biosciences, USA). 20% glycerol stock of the purified enzyme was stored at -20°C for further study.

#### Optimal condition of M3 alginate lyase activity

The effects of pH and temperature on purified M3 lyase activity were investigated using 0.2% sodium alginate solution as a substrate. After 20 µg purified M3 alginate lyase was incubated in 0.5 ml of 40 mM various buffer solutions with different pH for 10 min, the enzyme reaction was started by adding 0.5 ml of 0.4% substrate solution. For the study of temperature dependence, the enzyme reaction was carried out by incubation at different temperatures from 20°C to 80°C for 10 min. For control group, all steps were same except the enzyme denatured by heating was used.

For the study of the effects of metal ions, nine salt solutions were prepared to 2 mM in 20 mM phosphate buffer. 20 µg of purified enzyme was incubated in 0.5 ml salt solution for 10 min and then the reaction was started by adding 0.5 ml of 0.4% sodium alginate solution as a substrate.

#### Substrate specificity of M3 alginate lyase

The substrate specificity of M3 alginate lyase was investigated by using sodium alginate (3,500 CPS), polyG block, polyM block and polyMG block, starch, agar and agarose. Used polyG block and polyMG block were obtained according to Haug *et al* [10]. To obtain polyM block, sodium alginate was digested by polyG-specific lyase from previous study [15] and undigested alginate block was precipitated by adding ethanol to be final 50% concentration. The precipitate

was lyophilized and used as a polyM block.

Reaction products obtained from the mixture of M3 alginate lyase and substrate were analyzed by thin layer chromatography as described in previous paper [15]. 10 µg purified enzyme was mixed with 1 ml of 1% substrate in 20 mM phosphate buffer (pH8.0) and incubated overnight at 37°C. The degrading mixture which was desalted by Sephadex G-10 column (2.5 x 45 cm) was separated on the silica gel TLC plate by using 1-butanol-formic acid-water (4:6:1, v:v:v) as a solvent system.

## Results and Discussions

#### Overexpression of M3 alginate lyase gene in *E. coli* BL21 (DE3)

New alginate lyase gene of *Streptomyces* sp. M3 was elucidated at previous study [14]. M3 alginate lyase consisted of 780 nucleotide and 259 amino acid residues as shown as Fig. 1. Because signal peptide sequence was predicted as from <sup>1</sup>Met to <sup>35</sup>Ala (underlined sequence at Fig. 1), alginate lyase gene without signal peptide was cloned to pColdI expression vector for the enzyme overexpression. The Coomassie staining of 12% SDS-PAGE gel showed the overexpression of 28.5 kDa protein when the recombinant cell harboring pColdI/M3 lyase plasmid was cultured with IPTG (Fig. 2A lane 4). In the immunoblotting analysis, protein bands fused with (His)<sub>6</sub>-tag were detected at the same size whether the recombinant cells were cultured with IPTG or not (Fig. 2 lane 3 and lane 4). When M3 alginate lyase was blasted to GenBank database, M3 lyase exhibited to have highly sequence homology with other polyguluronate lyases which were belonged to polysaccharide lyase Family 7 (EC 4.2.2.11, 15,16). Family 7 lyase including Aly-PG lyase from *Corynebacterium* sp. ALY-1 which M3 lyase had 75% protein sequence identity with was reported to have β-jelly roll sandwich fold containing the active site region and lid structure [17].

#### Optimum condition of M3 alginate lyase

M3 alginate lyase exhibited the highest activity at pH 9.0 (Fig. 3). Tris-HCl buffer was more appropriate than phosphate buffer for the assay of recombinant M3 lyase. For 10 min assay, M3 alginate lyase was most active at 60°C than any other temperature (Fig. 4). ALG-5 alginate lyase (from *Streptomyces* sp. Strain ALG-5) without signal peptide which had difference at seven amino acid residues from overex-

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1 - ATGAGTCTCACCCGCAAGCGCAGTCTCGCCACCACCGGCGTGGCCGCACTCTCCGCCCTG - 60
1 - M S L T R K R S L A T T G U A A L S A L - 20
61 - GCCGCCCTGACCCTCCCGCTCACCCTGCGGGCACCGCCACCAGCGCCGCCCCCGTGGCAGC - 120
21 - A A L T L P L T T A G T A T A A A P C D - 40
121 - TACCCGCCCAGCAGCTCAACCTGACCAACTGGGAGGTAAACCCTGCCGACCGGCTCCAGC - 180
41 - Y P A Q Q L N L T N W K U T L P T G S S - 60
181 - GGCTCGCCACCGAGGTCAAGCAGCCCGCTCTCGGACCTTCTCGTCCAGCCCTGGTTC - 240
61 - G S P T E U K Q P A L A T F S S S P W F - 80
241 - ACCGTGAAGTCCAAGTGCACCGCGTCCAGTTCGGTGGCGGTCAACGCCGTCAAGACA - 300
81 - T U N S K C T G U Q F R S A U N A U T T - 100
301 - CCCAACTCCAGCTACGGACCGCCGAGCTGCGTGAGATGACCGACACGGGAACCGAGAAC - 360
101 - P N S S Y G R A E L R E M T D N G T E N - 120
361 - GCCTCCTGGTGGCGGACGTCCGGCACCACACCATGACCTTCGGGAGGCGTTCACCAAG - 420
121 - A S W S A T S G T H T H T F R E A F N K - 140
421 - CTCCCAACGACAGCCGACCGTGGTGGCGCCAGATCCACGACGGCGACGACGACGTCG - 480
141 - L P N D K P H U U G A Q I H D G D D D U - 160
481 - ACGGTCTTCCGCCTGGAGGGGACCAGCCTCTACATCACCAGGGGCGACGACCCACCAC - 540
161 - T U F R L E G T S L Y I T K G D D T H H - 180
541 - AAGCTGGTGACCAGCAACTACCAGCTGCACACGGTCTTCGAGGGCAAGTTCGTGTCAGC - 600
181 - K L U T S N Y Q L H T U F E G K F U U S - 200
601 - GCGGCCAGATCAAGGTCTACTACAACGGCGTCCCTCCAGACCACGATCCCGCACCCGCG - 660
201 - G G Q I K U Y Y N G U L Q T T I P H T A - 220
661 - TCCGGCACTACTTCAAGGCCGGCGGTACACCCAGGCCAAGTTCGGCAACTCGTCCCGG - 720
221 - S G N Y F K A G G Y T Q A N C G N S S P - 240
721 - TGCAGCAGCTCCAAGTACGGGCGAGTGACGATCTACAAGCTGCTGGTCAACCACTCCTGA - 780
241 - C S S S N Y G Q U T I Y K L L U T H S * - 260
    
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Fig. 1. The nucleotide sequence and deduced amino acid sequence of alginate lyase gene of *Streptomyces* sp. M3. Underlined region was predicted to be a signal peptide by SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>).

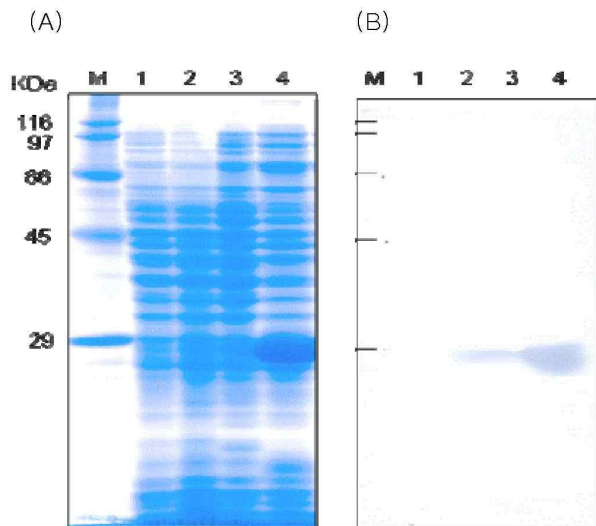


Fig. 2. SDS-PAGE and immunoblotting analysis of overexpressed alginate lyase of recombinant *E. coli*. *E. coli* BL21 was transformed with plasmid and cultured as described in previous paper (15). (A) Separated proteins on 12% SDS-PAGE gel was stained with Coomassie Blue R-250. (B) Electrotransferred proteins on NC paper was blotted with anti-(His)<sub>6</sub>-tag antibody. M: standard protein marker, lane 1 and 2: BL21 cells containing pColdI vector without and with IPTG induction, lane 3 and 4: BL21 cells containing pColdI/M3 alginate lyase gene without and with IPTG induction.

pressed M3 lyase showed highest activity at optimum temperature 50°C and optimum pH 8 [21]. When M3 lyase was incubated with 1 mM of various salt solutions, Mn<sup>++</sup> and Ca<sup>++</sup> increased enzyme activity more than 2 folds but Mg<sup>++</sup>,

Co<sup>++</sup>, Na<sup>+</sup>, K<sup>+</sup>, and Ba<sup>++</sup> showed no significant difference (Fig. 5). Hg<sup>++</sup> and Zn<sup>++</sup> inhibited the activity of M3 alginate lyase completely.

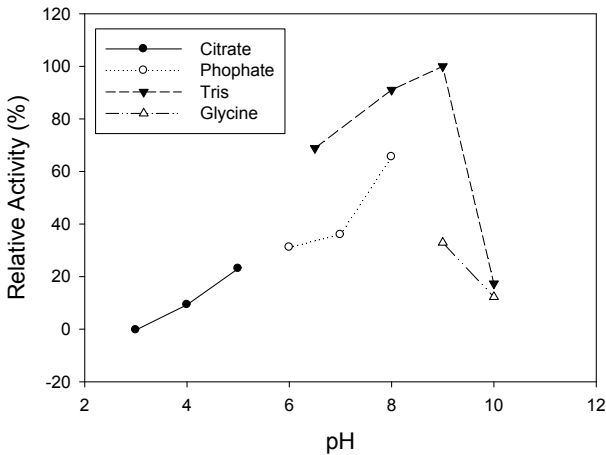


Fig. 3. pH dependency on M3 alginate lyase activity. The enzyme reaction was started by adding 0.5 ml of 0.4% alginate solution after 20 µg of purified M3 lyase was incubated in 0.5 ml various buffers with different pH for 10 min. The relative enzyme activity was calculated compared to the activity at pH 9.0.

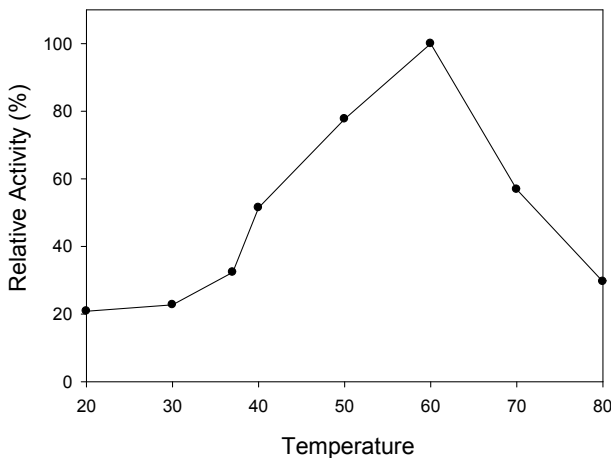


Fig. 4. Temperature dependency on M3 alginate lyase activity. The enzyme reaction was started by adding 20 µg of purified M3 lyase in 1 ml of 0.2% alginate solution in 20 mM phosphate buffer which incubated at indicated temperature. The reaction was carried out for 10 min.

Substrate specificity of M3 lyase

The substrate specificity of M3 alginate lyase was analyzed by incubating enzyme with 0.2% substrate solution for 10 min at 37°C (Fig. 6). The M3 lyase could degrade alginate with high molecular weight, polyMG block and polyG block better than polyM block. The positive activity on polyM block was probably due to its contents of guluronate residue (8.8% guluronate contamination in polyM block). This data shows that M3 alginate lyase is a polyguluronate lyase which seems unable to cleave M-M

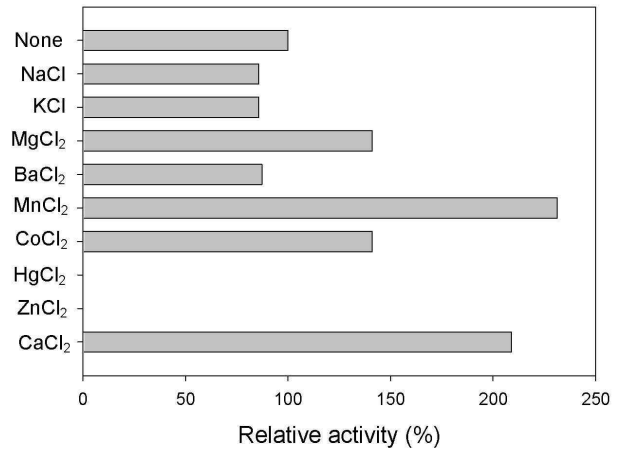


Fig. 5. Metal ion dependency on the activity of M3 alginate lyase. The enzyme reaction was started by adding 0.5 ml 0.4% alginate solution in buffer into the mixture of M3 alginate lyase and salt solution. Before adding alginate solution, 20 µg of purified enzyme was incubated in 0.5 ml 2 mM metal ions in 20 mM phosphate buffer for 10 min. Relative activity was compared with the degrading activity of M3 lyase when no metal ion was included.

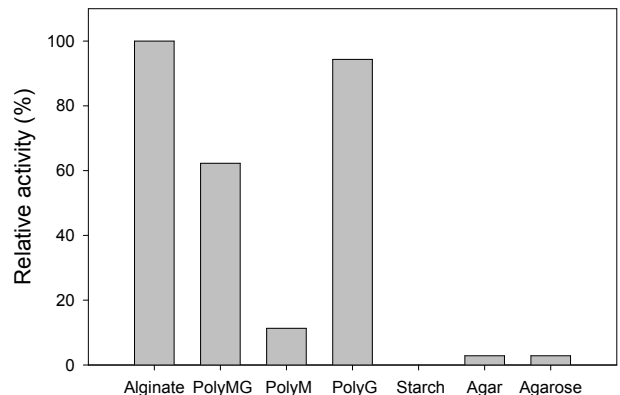


Fig. 6. Substrate specificity of M3 alginate lyase. 20 µg of purified enzyme was reacted with 0.2% each substrate solution at 37°C for 10 min. Used polyMG and polyG block was made by acid hydrolysis and polyM block was made by digestion with polyG-specific lyase and ethanol precipitation. Relative activity was compared with the degrading activity of M3 lyase on 0.2% alginate solution.

bonds. The enzyme activity on polyMG block could be due to cleavage either G-G bond or G-M bond present in polyMG block. TLC analysis of reaction products showed the degradation profile of M3 alginate lyase as Fig. 7. Oligoalginate obtained by incubating alginate or polyG block with M3 lyase consisted to dimer, trimer and tetramer with almost same portion. TLC data also showed that M3

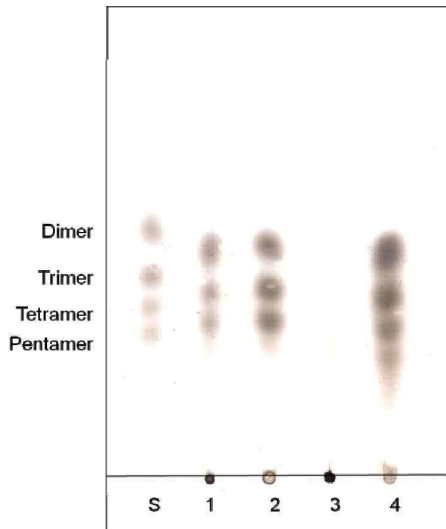


Fig. 7. Degradation profile of M3 alginate lyase on TLC plate. Purified M3 lyase was incubated in 0.2% substrate solution (1: alginate, 2: polyG block, 3: polyM block, 4: polyMG block) overnight. The reaction products were desalted by Sephadex G-10 column and freeze-dried. Samples were spot on TLC plate and developed in 1-butanol-formic acid-water mixture (4:6:1). TLC was visualized after spaying with 10% sulfuric acid in ethanol and heating at 105°C for 10 min. Standard (S) is the oligoalginate mixture purified from Bio-Gel P2 column chromatography [15].

alginate lyase could not product any significant alginate oligomers from polyM block. Oligomer products from polyMG block were dimer, trimer, tetramer and pentamer. PolyMG block used in this experiment had MG ratio 0.91 (M/G) and MG order was not known.

Fig. 8 showed the protein sequence alignment of M3 lyase and ALG5 lyase cloned in this lab [15]. From phylogenetic tree of 16S rDNA, marine bacterium *Streptomyces* sp. ALG-5 harboring ALG5 lyase was closest to *Streptomyces parvus* and *Streptomyces badius* [15] but the closest relatives of *Streptomyces* sp. M3 were *Streptomyces griseorubens* and *Streptomyces pseudogriseolus* subsp. *Glucofermentans* [14]. Eleven amino acid residues were different and one amino acid was deleted in ALG5 lyase. But R, Q, H, Y and Y which are presumed to form active site were conserved [14,15]. Although many polyguluronate lyases elucidated to classified into Family 7, hypothetical *Pseudomonas aeruginosa* protein PA1167 with a  $\beta$ -jelly sandwich fold like Family 7 lyase was reported to degrade polyMG block (100%) preferentially more than polyM block (16.1%) and polyG block (1.83%) [24]. Homology modeling showed that both M3 lyase and ALG5 lyase also had  $\beta$ -jelly roll sandwich fold [14,15]. Site directed mutation of M3 lyase for enhancing the degrading activity and stability is on studying.

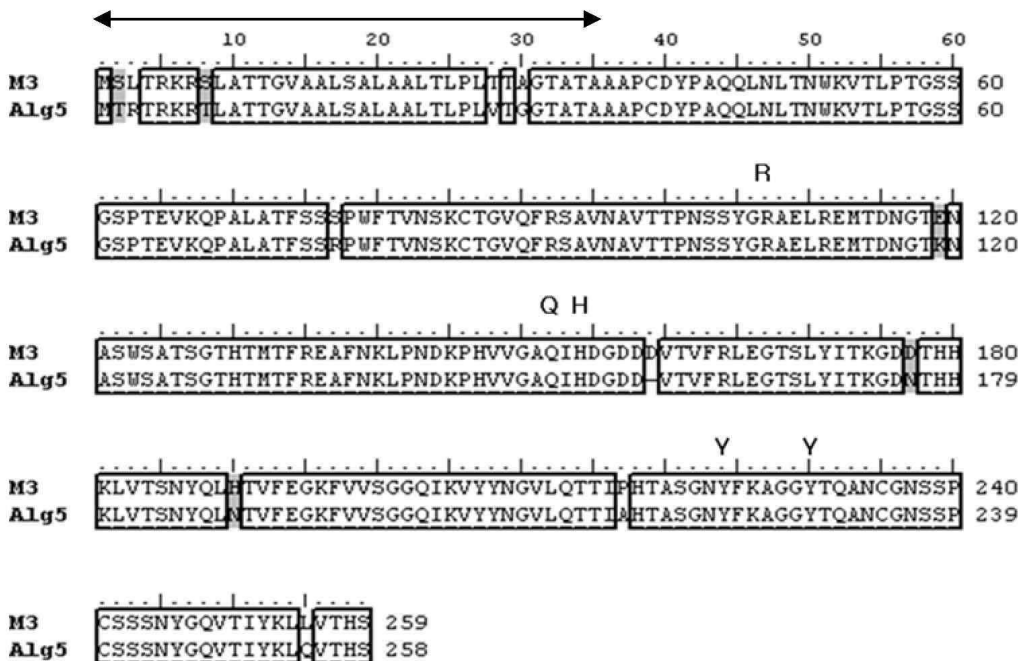


Fig. 8. Comparison of protein sequence between M3 alginate lyase to ALG5 lyase. The arrow from <sup>1</sup>Met to <sup>35</sup>Ala indicates the signal peptide sequence. Both M3 lyase and ALG5 lyase were overexpressed without signal peptides. Bold letters above the sequences, R, Q, H, Y and Y are the residues that are presumed to form an active center.

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초록 : 해양세균 *Streptomyces* sp. M3로 부터 얻은 재조합 polyG-specific lyase의 특성

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전 연구에서 해양세균 *Streptomyces* sp. M3의 새로운 alginate 분해효소를 signal peptide가 제거된 상태로 클로닝하고 *E. coli* BL21 (DE3) 균에 형질전환시켰다. 본 연구에서는 배양할 때 IPTG를 첨가하여 M3 alginate 분해효소 단백질을 과발현시키고 Ni-Sepharose 친화력 chromatography로 정제하여 생화학적 성질을 조사하였다. 기질특이성을 시험하기 위한 235 nm에서의 흡광도와 TLC 분석 결과로부터 M3 alginate 분해효소가 polyG block에 기질특이성을 나타냄을 알 수 있었다. M3 분해효소를 기질과 10분 동안 반응시켰을 때, 최적 pH 및 최적온도는 pH 9 및 60°C 이었다. 1 mM  $Ca^{++}$  및  $Mn^{++}$ 은 alginate 분해활성을 2배 증가시킨 반면  $Hg^{++}$  및  $Zn^{++}$ 는 분해활성을 완전히 저해하였다.  $Mg^{++}$ ,  $Co^{++}$ ,  $Na^+$ ,  $K^+$ , 및  $Ba^{++}$ 은 M3 alginate 분해효소의 활성화에 거의 영향을 미치지 않았다.