Journal of Microbiology and Biotechnology
Vol. 5, No. 2, 92-95, 1995

Alginate Lyase Production of Halophilic *Pseudomonas* sp. by Recombinant *Escherichia coli*

KONG, IN-SOO[1]*, YOUNG-OK KIM[1], JIN-MAN KIM[2], SUNG-KOO KIM[1], DOO-HWAN OH[3], JU-HYUN YU[3] AND JAI-YUL KONG[1]

Department of Biotechnology and Bioengineering, National Fisheries University of Pusan, Pusan 608-737[1], Korea
Department of Biotechnology, Yosu National Fisheries University, 195 Kuk-dong, Yosu 550-749[2], Korea
and Bioproducts Research Center, Yonsei University, 134 Shichon-dong, Sudaemoon-ku, Seoul 120-749[3], Korea

Halophilic *Pseudomonas* sp.W7 isolated from laver in the southern sea of Korea showed alginate lyase activity. Gene (aly) encoding alginate lyase was cloned in *E.coli* JM83 and the N-terminal amino acid sequence of the enzyme was determined after purificaion. The recombinant enzyme has been shown to have a molecular weight of about 40kDa after 12% SDS-polyacrylamide gel electrophoresis.

Alginate is a commercially important algal polysaccharide which occurs in the cell wall and intercellular mucilage in phaeophycean algae. It has been used widely for a broad range of applications and many facets of the properties of this polysaccharide have been exploited. In industry, alginates are used as emulsifiers and thickners in foods, cosmetics, pharmaceuticals and as supports for the immobilization of cells or enzymes. Alginate is glycuronan composed of β-D-mannuronate and α-L-guluronate (4). Alginate can be degraded by a group of enzymes that catalyze the β-elimination of the 4-O-linked glycosidic bond (3). Alginate lyase isolated from molluscs generally showed a preference for mannuronate-rich substrate (9, 11).

However, several bacterial extracellular enzymes preferred either mannuronate or guluronate blocks in the alginate molecules (5, 6). In an earlier study, the guluronate specific algiante lyase from *Klebsiella pneumoniae* was cloned using the cosmid vector pMMB 33 in *E.coli* (2). Recently, the cloning and sequencing of alginate lyase into a variety of bacteria have been reported (1, 7, 13, 14).

In this report, cloning and expression of the gene in *E.coli* as well as purification of the cloned product were carried out to evaluate the molecular information of alginate lyase gene from halophilic *Pseudomonas* sp. W7.

*Corresponding auter
Key words: Alginate lyase, halophilic *Pseudomonas* sp.,

MATERIALS AND METHODS

Bacterial Strains and Media

Halophilic *Pseudomonas* sp. W7 isolated from laver in the southern sea of Korea was used as the source of alginate lyase. The alginate lyase activity of the isolated strain was detected with a solution of 0.05% (w/v) ruthenium red on agar plate containing 0.5% alginate. *Psuedomonas* sp. W7 was maintained on modified marine medium (peptone 5g, glucose 10g, Na_2HPO_4 0.008g, NH_4NO_3 0.0016g, NaCl 23.4g, KCl 0.7g, $MgCl_2 \cdot 6H_2O$ 10.6g, $CaCl_2$ 1.1g, Na_2SO_4 3.9g, $NaHCO_3$ 0.2g, $(NH_4)_2SO_4$ 1.0g, K_2HPO_4 0.01g, Tris 6.05g, pH 7.8, D.W 1*l*). Cultivation was done at 25°C for 48h. *E.coli* JM83 and JM109 were used as the host strain.

Isolation of DNA

Pseudomonas sp. W7 cells were harvested at the end of the exponential growth phase. Chromosomal DNA was prepared by the method of Caswell *et al.* (2). For rapid isolation of plasmids from *E.coli,* the alkaline lysis method was used.

Molecular Cloning

General methods were as described by Sambrooks *et al.* (12). Recombinant transformants were screened for alginate lyase activity on alginate agar flooding with 1% $CaCl_2$ in 70% ethanol. The formation of visible halos around the colonies indicated the production of alginate lyase. DNA sequencing was done by the dideoxy chain

termination method.

Enzyme Assay

Alginate lyase activity was determined by absorbance of reaction mixture at 235nm (8). The reaction mixture was incubated at 28°C for 40min. Plate assay was performed after exposure to dessicator saturated with chloroform for 1h. One unit of acitivity was defined as the increase of absorbance by 0.01 at 235nm per min. Specific activity was defined as units per mg of protein.

Enzyme Purificaiton

The purification was carried out at 0~4°C. Cell pellet from 1 liter of culture broth was resuspended in the 50mM phosphate buffer (pH 7.5) and then disrupted by a sonicator. The cell debris was removed by centrifugation at 8,000g for 30min. The supematant obtained was applied to a DEAE-Sepharose CL-6B column (2× 15cm) equilibrated with 50mM phosphate buffer (pH 7.5) and the protein was eluted by 40mM NaCl in the same buffer. The alginate lyase-containing fractions were combined and put on a Mono Q, HR 5/5 anion exchange HPLC. The absorbed proteins were eluted with a continuous linear gradient of 0~0.2M NaCl in 50mM phosphate buffer (pH 7.5) at flow rate of 0.2 ml/min. The protein content of eluate was monitored by UV detector at 280nm.

NH_2-terminal Amino Acid Sequence Analysis

N-terminal amino acid sequence of the purified enzyme was determined by automated Edman degradation using a gas phase automatic sequencer (Milligen 6600B).

Localization of Alginate Lyase in *E.coli*

Fractionation of extracellular, periplasmic, and intracellular alginate lyase was performed by the osmotic shock method (10).

RESULTS AND DISCUSSION

Cloning of Alginate Lyase Gene

To construct a genomic library, the chromosomal DNA of *Pseudomonas* sp. W7 was digested with *Hind*III and ligated with pUC9. After transformation into *E.coli* was performed, a stable alginate lyase positive clone was screened among 10,000 transformants. The analysis of the plasmid DNA digested with *Hind*III fragment in pUC9 which was designated as pKAL26. To further localize the essential region of the alginate lyase gene in the 4.3kb insert, plasmid pKAL26 was digested with *Hind*III and *Dra*I. 2.3kb fragment was isolated from agarose gel and these fragments were mixed with *Hind*III-*Sma*I digested pUC9. A transformant, carrying 2.3kb insert DNA, showed activity and was designated as pKAL24 (Fig. 1).

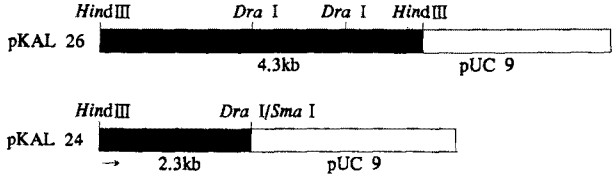

Fig. 1. Schematic representation of plasmid construction
→: Partial sequenced fragment.

Identification of Alginate Lyase Produced in *E.coli*

For the distribution of alginate lyase, *E.coli* JM83 harboring plasmid was cultured in LB broth and enzyme activity was assayed. The alginate lyase activity was

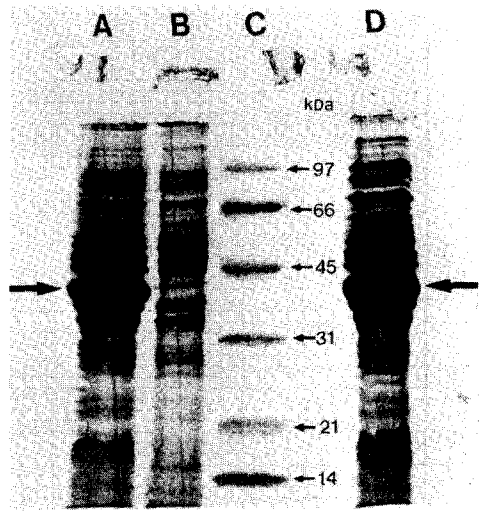

Fig. 2. SDS-PAGE analysis of recombinant alginate lyase in *E.coli*
Lane A: cell extract from *E.coli* JM83 (pKAL 26), Lane B: cell extract from *E.coli* JM83 (pUC 9), Lane C: standard molecular weight, lysozyme: 14kDa, trypsine inhibitor: 21kDa, avalbumin: 45kDa, bovine serum albumin: 66kDa, phosphorylase b: 97kDa, Lane D: cell extract from E.coli JM83 (pKAL 24)

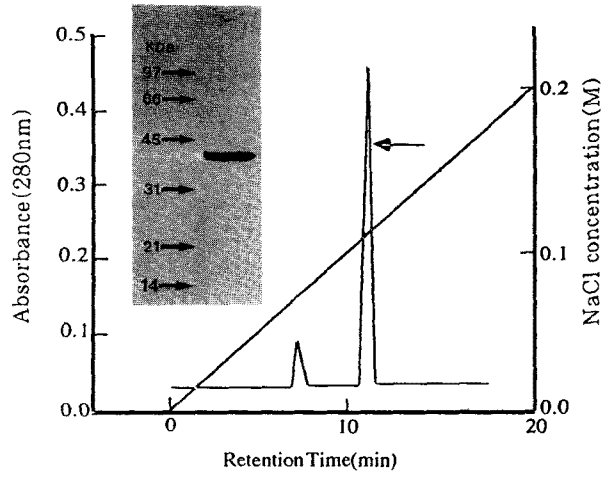

Fig. 3. HPLC analysis of alginate lyase with Mono Q column.
The inset shows the purified alginate lyase by 12% SDS-PAGE

Table 1. Purification of the enzyme in E.coli JM83 (pKAL 24)

Purification Step	Total Protein (mg)	Total activity (unit)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell extract	42	4704	112	1.0	100
DEAE Sepharose	6.7	1200	179	1.5	26
Mono Q HPLC	4.1	820	200	1.8	17

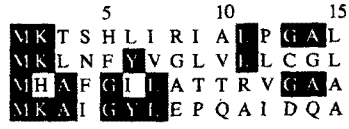

Fig. 4. Comparison of N-terminal amino acid sequence of alginate lyase.
P.aeruginosa from Boyd *et al.* (11), *P.* sp. OS-ALG-9 from Maki *et al.* (10). Strain A1 from Yonemoto *et al.* (9)

founded mostly in the cytoplasmic fraction (98%) and a small amount of enzyme activity was detected in a periplasmic space. (data not shown) To identify the protein from the recombinant plasmid, 100μl of cell culture was harvested by centrifugation. The pellet was suspended in 20μl of SDS buffer and analyse in 12% SDS-PAGE. Over expressed gene product band was observed, suggesting that alginate lyase had a molecular weight of 40kDa (Fig. 2).

Purification of Recombinant Alginate Lyase from *E. coli*

The recombinant alginate lyase was purified from the cell extracts. The crude enzyme was applied to the DEAE Sepharose CL-6B column. Active fractions were pooled and dialyzed with 50mM phosphate buffer (pH 7.5). The alginate lyase was purified to homogeneity with anion exchange HPLC using a Mono Q column. Two peaks were identified at retention time, 8 min and 11min, respectively. However, enzyme activity was obtained from the main peak (retention time; 11min). The purity was analyzed by 12% SDS-PAGE and a single band was detected (Fig. 3). The results of the overall purification procedures are summarized in Table 1.

NH_2-terminal Amino Acid Sequence

Expression of the alginate lyase gene in *E.coli* cells appeared 40kDa of molecular weight on SDS-PAGE gel. The molecular weight of other alginate lyase previously reported were 46kDa from *Pseudomonas* sp. OS-ALG-9(7) and 63kDa from unidentified bacterium (14), respectively. However, the molecular size of gene product in *E.coli* is very close to 41KDa alginate lyase of *Pseudomonas aeruginosa* (13).

The N-terminal amino acid sequence was determined and compared with those of other alginate lyase (Fig. 4). The homology is very low and proves to be different from those which were published previously. The N-terminal amino acid sequence of purified protein was proved as Met-Lys-Ala-Ile-Gly-Tyr-Leu-Gln-Pro-Glu-Ala-Ile-Asp-Glu-Ala. This result is matched to the deduced amino acid sequence from partial DNA sequence which is 5'- ATGAAAGCCATCGGTTACCTAGAGCCTCAAGC-CATCGATCAAGCA-3'. However, the complete DNA sequence of this gene must be known to discuss protein structure and function.

Acknowledgement

This study was supported in part by Non-Directed Research Fund, Korea Research Foundation (1993) and Basic Science Research Institute Program, Ministry of Education (1994), Project No.BSRI-94-4410.

REFERENCES

1. Boyd, A.Ghosh, M.May, T.B.shinabarger, D.Keogh, R, and Chakrabarty, A.M. 1993. Sequence of the alg L gene of *Pseudomonas aeruginosa* and purification of its alginate lyase product. *Gene* **131**: 1-8.
2. Caswell, R.C., Gacesa, P., Lutrett, K.E. & Weightman, A.J. 1989. Molecular cloning and heterologous expression of a *Klebsiella pneumoniae* gene encoding alginate lyase. *Gene* **75**: 125-134.
3. Gacesa, P. 1987. Alginate-modifying enzymes. *FEMS Lett.* **212**: 199-202.
4. Haug, A.and Larsen, B. 1962. Quantitative determination of the uronic acid composition of alginate. *Acta. Chem. Scand.* **16**: 1908-1918.
5. Hisano, T.Nishimura, M.Y onemoto, Y. Abe, S. Yamashita, T. Sakaguchi, K.Kimura, A. and Murata, K. 1993. Bacterial alginate lyase highly active on acetylated alginate. *J. Ferment. Bioeng.* **75**: 332-335.
6. Jeffrey, J.B., Doubet, R.S. and Ram1, J. 1984, Alginase enzyme production by *Bacillus circulans. Appl. Environ. Microbiol.* **47**: 704-709.
7. Maki, H., Mori, A., fujiyama, K., Kinoshita, S., Yoshida, T. 1993. Cloning, sequence analysis and expression in *Escherichia coli* of a gene encoding an alginate lyase from *Pseudomonas sp.* J OS-ALG 9. *Gen. Microbiol.* **139**: 987-993.
8. Muramatsu, T. and Sogi, T. 1990. Characterization of alginate lyase from marine bacterium. *Comp. Biochem.* **97**: 103-108.
9. Nakada, H.I.and Sweeny, P.C. (1967). Alginic acid degradation by eliminases from *abalone hepatopancreas. J.*

Biol. Chem. **242**: 845-851.
10. Neu, H.C. and Heppel, L.A. 1965. *J.Biol, Chem.* **240**: 3685-3692.
11. Nishizawa, K., Fujibayashi, S. and Kashiwabara, S. 1968. Alginate lyase in the hepatopancreas of a marine molluc *Dolabela auricula. J.Biochem.* **64**: 25-37.
12. Sambrook, J.Fritsch,. E.F. and Maniatis, T. 1982. *Molecular cloning: a laboratory manual,* 2nd ed., Cold Spring Harbor Laboratory, N.Y.
13. Schiller, N.L.Monday, S.R.Boyd, C.M.Keen, N.T. and Ohman1, D.E. 1993. Characterization of the *Pseudomonas aeruginosa* alginate lyase. *J. Bacteriol.* **175**: 4780-4789.
14. Yonemoto, Y.Tanaka, H.Hisano, T.Sakaguchi, K.Abe, S. Yamashita, T.Kimura, A. and Murata, K 1993. Bacterial alginate lyase gene: Nucleotide sequence and Molecular route for generation of alginate lyase species. *J. Ferment. Bioeng.* **75**: 336-342.

(Received February 16, 1995)