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NOTE

Nucleotide Sequence Analysis and Expression of the Alginate Lyase Gene from *Pseudomonas* sp. W7 in *Escherichia coli*

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Abstract The gene encoding alginate lyase was isolated from a library constructed with the vector, pUC19, and expressed in *Escherichia coli*. The nucleotide sequence of the cloned alginate lyase gene (*ALY*) from *Pseudomonas* sp. W7 was determined. The nucleotide sequence revealed a 1, 035 bp open reading frame (ORF), encoding 345 amino acid residues with a calculated molecular mass of 37,478 Da. The N-terminal amino acid sequences (15 residues) of purified alginate lyase corresponded to that of the deduced amino acid sequence.

Key words: *Pseudomonas*, alginate lyase, nucleotide sequence

Alginate is an important algal polymer, which is comprised of β-D-mannuronic acid and α-L-guluronic acid. Alginates are used as emulsifiers, thickeners in foods, components of cosmetics, and supporters for the immobilization of cells or enzymes. Alginate can be degraded by a number of enzymes that catalyze the degradation of alginate by a β-elimination of the 4-*o*-linked glycosidic bond with formation of unsaturated uronic acid containing oligosaccharides [2]. Alginate lyases have been isolated from various microorganisms such as *Pseudomonas aeruginosa* [14], *Bacillus circulans* [3], *Enterobacter cloacae* M-1 [11], *Klebsiella aerogenes* [10], and *P. alginovora* [1]. Alginate lyases from microorganism have been characterized to investigate the structural properties and the functions of alginate. Recently, the cloning and sequencing of alginate lyase genes from other *Pseudomonas* sp. have been reported [1, 8, 14]. We have previously reported the isolation of alginate degrading bacterial strain *Pseudomonas* sp. W7 and characterization of the purified recombinant alginate lyase, which was cloned in *E. coli* [5, 7]. In this paper, we report the nucleotide sequence of the alginate lyase gene from *Pseudomonas* sp. W7.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Cultivation

E. coli JM83 harboring pKAL 24 plasmid was used in all experiments [7]. *E. coli* was grown at 37°C in Luria-Bertani (LB) broth (tryptone 1%, yeast extract 0.5%, sodium chloride 0.5%) containing ampicillin (50 μg/ml).

DNA Sequencing

The nucleotide sequence of alginate lyase gene was determined using the dideoxy chain termination method with an automatic DNA sequencer (ABI PRISM model 377 Perkin Elmer Co., Ltd., U.S.A.). The reverse primers and forward primers were synthesized on the exact sequence of templates. DNA and protein sequences were analyzed by using DNASIS program (Hitachi Software Engineering Co., Ltd., Tokyo)

Enzyme Assay

Alginate lyase activity was determined by absorbance of the reaction mixture at 235 nm by detection of the unsaturated nonreducing terminus [13]. To prepare the enzyme solution, the whole cell was disrupted with ultrasonic treatment (at 9 kHz, 4°C). The cell extract was centrifuged at 10,000×g, 4°C, for 20 min. Clear supernatant was used for the enzyme solution. Enzyme solution was incubated with 0.1% sodium alginate in 50 mM phosphate buffer (pH 7.5) at 28°C, for 40 min. One unit of activity was defined as an increase of absorbance of 0.001 at 235 nm per min.

Zymography

To identify the active band, zymography was performed by the method of Pecina *et al.* [12]. Cell extracts were

prepared as mentioned above. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by Laemmli method [9] with 12% slab gel containing sodium alginate (2 mg/ml). After electrophoresis, the gel was renatured by soaking 3 times in 50 mM Tris-Cl (pH 8.0), 1% (w/v) casein, 2 mM EDTA, 0.01% (w/v) sodium azide at 4°C, for 30 min, and incubated in 10 mM phosphate buffer (pH 7.0), containing 200 mM NaCl, 200 mM KCl, 0.01% (w/v) sodium azide at 30°C, for 12 h. The gel was stained with a solution of 10% (w/v) cetylpyridinium chloride for 20 min at room temperature to detect the alginate-degrading band.

RESULTS

Expression of Alginate Lyase in *E. coli*

Figure 1 shows the growth curve of *E. coli* (pKAL 24) and the alginate lyase activity during the cell growth. The enzyme activity was changed with cell growth associated pattern. The maximum activity was reached after 9 h. The activity of alginate lyase was 1 U per µg protein at 9 h and decreased to 0.8 U/µg at 12 h. Alginate lyase activity increased through the exponential phase of cell growth and decreased at the stationary phase. Thereafter, the enzyme activity began to decrease. SDS-PAGE analysis of the cell extract from cultures of 6 h, 12 h, 18 h, and 24 h showed that the production of alginate lyase was slightly decreased from 12 h. (Fig. 2).

Zymography of Recombinant Alginate Lyase

A single active band at about 37 kDa region, which is the expected alginate lyase, was shown in the zymogram (Fig. 3B). To investigate whether the alginate could act as an inducer for the expression of the alginate lyase gene, alginate was added to the growth medium, and the productivity of the enzyme was measured by SDS-PAGE. The addition of alginate did not affect the productivity of alginate lyase (Fig. 3A). This result implies that the expression of alginate lyase gene might be constitutive in *E. coli.*

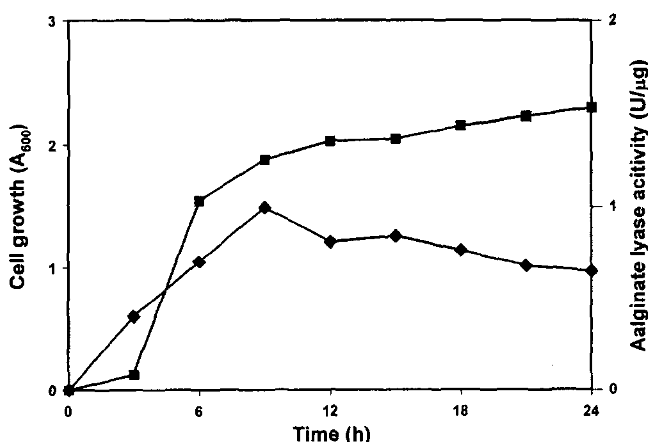

Fig. 1. Growth and alginate lyase activity of recombinant *E. coli* harboring pKAL 24 according to culture time.

The cells were grown in LB medium supplemented with ampicillin (50 µg/ml) at 37°C. ◆; cell growth, ■: enzyme activity.

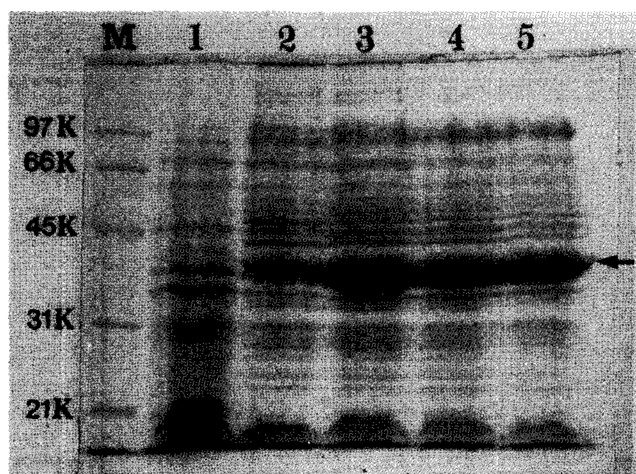

Fig. 2. SDS-polyacrylamide gel (12% slab gel) electrophoresis analysis of the recombinant alginate lyase.

Whole cell was resuspended in gel loading buffer and heated at 100°C, for 10 min. After electrophoresis, the gel was stained with Coomassie Brilliant blue R-250. Lane 1, *E. coli* JM83 (pUC 19); lane 2, 6 h cultured; lane 3, 12 h cultured; lane 4, 18 h cultured; lane 5, after 24 h cultured *E. coli* JM83 (pKAL 24).

Nucleotide Sequence of Alginate Lyase Gene

The nucleotide sequence of the alginate lyase gene (*ALY*) and the deduced amino acid sequence are presented in

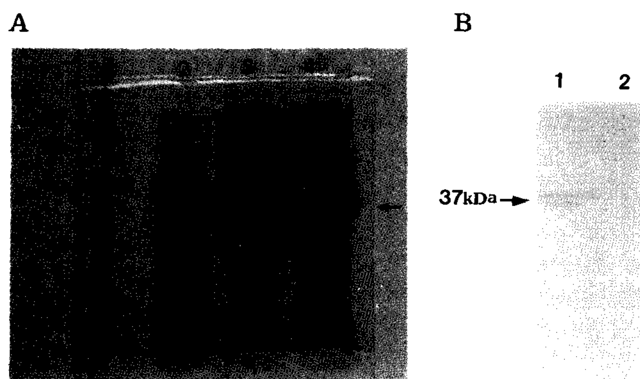

Fig. 3. The SDS-PAGE of the cell extract of *E. coli* (pKAL 24).

A. Lane 1, molecular weight marker (BioRad low range molecular marker); Lane 2, cell extract from *E. coli* JM83 (pUC 19); lane 3, cell extract from *E. coli* JM83 (pKAL 24); lane 4, cell extract of *E. coli* JM 83 (pKAL 24) cultured with 0.5% sodium alginate. B. Zymogram of *E. coli* JM83 (pKAL 24). Lane 1, cell extract of *E. coli* JM83 (pKAL 24); lane 2, cell extract from *E. coli* JM83. Arrows indicate alginate degrading band located at 37 kDa.

Fig. 4. This nucleotide sequence contains a single open reading frame of 1,035 nucleotides. A putative Shine-Dalgarno sequence (SD) and two possible promoter regions are found upstream from the ATG initiation codon. The alginate lyase gene contains two inverted repeat sequences between the SD sequence and two possible promoter sequences. The first possible promoter is located at -134 to -129: TTGTCA (-35 region); -111 to -106: TATTGT (-10 region) and the second possible promoter is located at -94 to -89: TTTATA (-35 region); -71 to -66: AATAAT (-10 region) from the initiation ATG. The putative promoter region of -134~-106 has a one base difference for the -35 region and two bases difference for the -10 region, when compared to the consensus promoter sequence of *E. coli*. However the -94 ~ -66 region has three bases difference for the -35 region and one base difference for the -10 region. The spacer between -35 and -10 hexamers is 18 bps. The putative SD sequence, AGAGGA, is located 6 bp from initiation ATG. We did not find a putative transcriptional terminator sequence in the downstream of the stop codon. This ORF encodes a protein of 345 amino acids having a molecular weight of 37,478 Da from DNASIS program. This value is in close proximity to the molecular weight predicted by SDS-PAGE. The predicted pI is 5.56 and the N-terminal 15 amino acids from the deduced polypeptide sequence exactly corresponds to that of the protein sequence previously reported [7]. This protein showed no significant homology with other alginate lyases from several *Pseudomonas* sp.

The Deduced Amino Acid Sequences of Alginate Lyase Gene

The codon usage of alginate lyase was analyzed. The G+C content of the alginate lyase gene (*ALY*) is 54%. The G+C content was increased to 61.5% in the third position. We did not find the codon of CGT (Arg), TGT (Cys), TGC (Cys) and AGG (Arg). The most frequently used codon is GCC (Ala). The deduced amino acid sequences have one AGA for arginine, which is rare in *E. coli* [6]. The amino acid composition of alginate lyase was analyzed. The values of Ala, Arg, Asx (Asp+Asn)

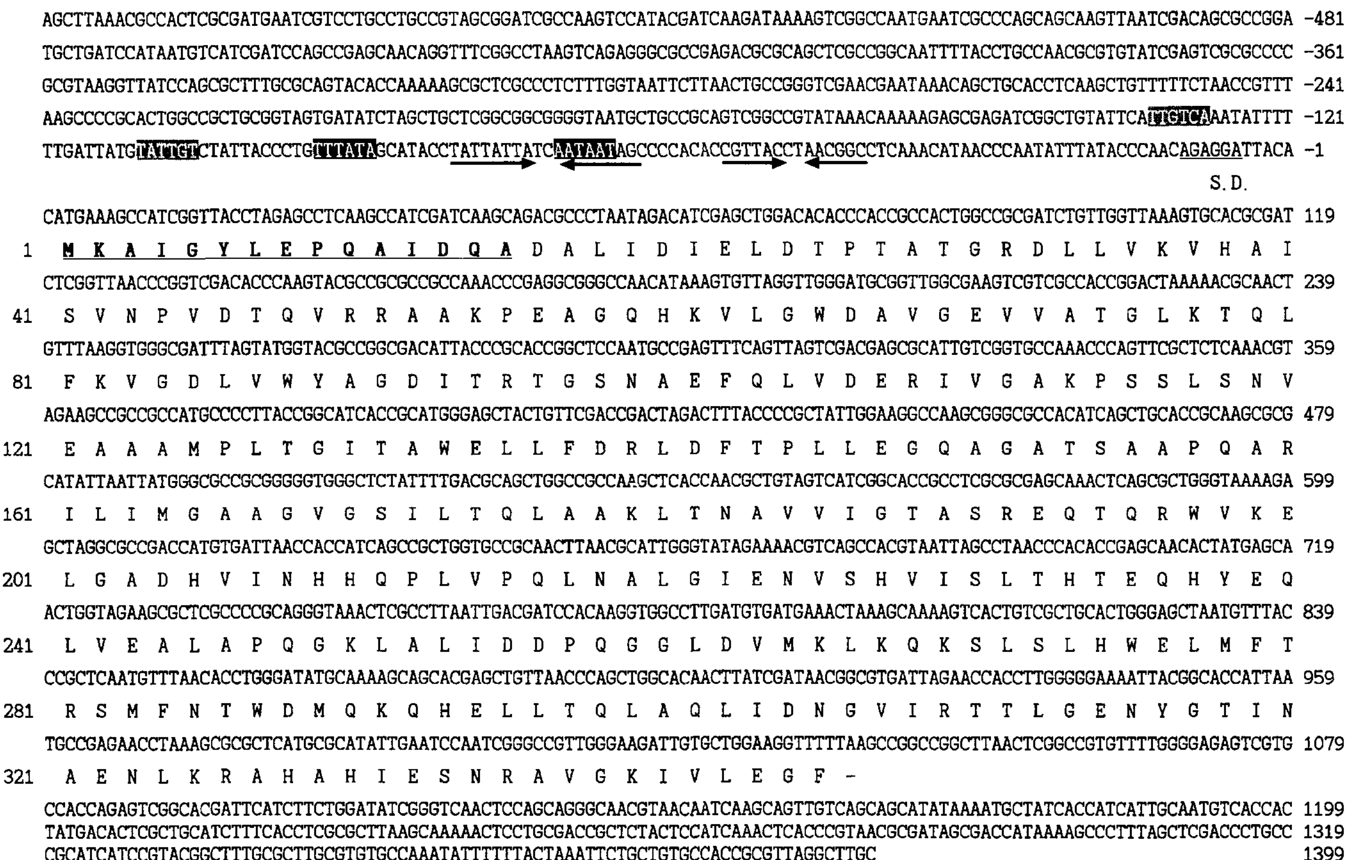

Fig. 4. Nucleotide sequence and deduced amino acid sequence of the alginate lyase gene.

The coordinate of nucleotide sequence is numbered to the right side and that of the deduced amino acid sequence to the left side. The -10 and -35 regions of two putative promoter sequences are represented by a shaded box. One pair of arrows shows an inverted repeat sequence. The N-terminal 15 amino acids are underlined. The nucleotide sequence of alginate lyase gene has been deposited in the GenBank DNA database under accession number, AF 050114.

and Glx (Glu+Gln) of the amino acids were relatively high, while those of Met, Trp, Tyr were low. The cysteine residue was not found in this amino acid sequence. The hydropathy of the deduced amino acid was calculated by the method of Hopp and Woods [4]. The hydropathy plot of this deduced amino acid sequence presented a hydrophilic feature. The average hydropathy value is -0.08. The N-terminal and C-terminal of this alginate lyase are relatively hydrophilic.

DISCUSSION

Although the alginate lyase is expressed with a large amount in *E. coli*, we could not find the inclusion body in the cell by electron microscopy (data not shown). In our previous studies, alginate lyase could be purified without any reducing agents such as guanidine hydrochloride or urea [7]. Hydrophobic interaction and disulfide bonding are known as reasons for the aggregation of the protein [15]. From the amino acid sequence analysis of alginate lyase of *Pseudomonas* sp. W7, it showed a hydrophilic profile (Fig. 5) and the deduced amino acid sequence does not have a cysteine residue. These might be reasons that this alginate lyase keeps a soluble state in the cell. Recently, the nucleotide sequences of alginate lyase genes from *Pseudomonas* sp. were reported. The reported alginate lyases from *Pseudomonas* sp. have various molecular masses. Crude enzymes of *Pseudomonas* sp. OS-ALG-9 showed four (90, 72, 60, and 54 kDa) distinctive alginate lyase activity bands identified by the zymogram [8]. *Pseudomonas alginovora* showed the 24 kDa of alginate lyase [1]. Alginate lyase of *Pseudomonas aeruginosa* was 41 kDa from the nucleotide sequence [14]. However, *Pseudomonas* sp. W7 isolated from marine environment produced the 37 kDa alginate lyase. In our previous studies, we reported on the promoter region of the alginate lyase gene and some features of purified alginate lyase from *Pseudomonas* sp. W7 [5, 7]. As shown in Fig. 2., *E. coli* (pKAL 24) produced very a high amount of alginate lyase. Alginate lyase was not overproduced in *Pseudomonas* sp. W7 like other *Pseudomonas* sp. [1, 8, 14]. When the *ALY* promoter of this study was removed and the structural gene was connected with the *lac* promoter, the expression level was markedly decreased in *E. coli* [5]. This result indicated that the unique feature of this promoter is devoted to expression of alginate lyase in *E. coli*. The production of alginate lyase was maximum at the late exponential phase of cell growth and decreased slightly during the cell growth. Catabolite repression of this promoter region was already reported [5]. However, more studies are needed to clarify that the decrease of production is only the result of the stability of the protein or of another repression for regulation in gene expression.

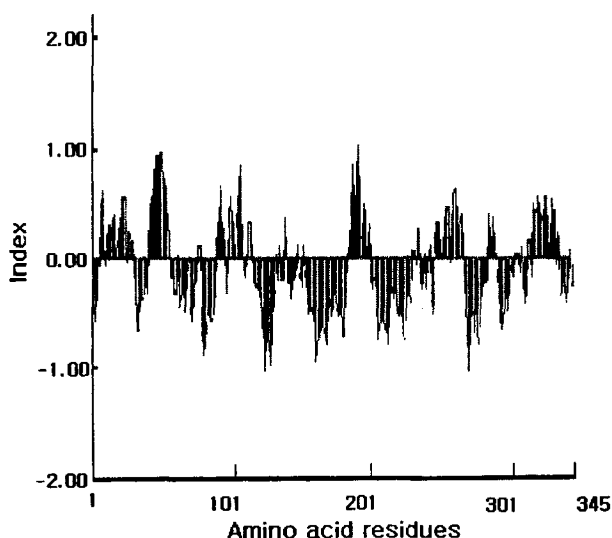

Fig. 5. Hydropathy analysis of corresponding protein of alginate lyase gene.

Hydropathy was calculated by the method of Hopp and Woods [4] with window size of 11 amino acid residues.

Acknowledgments

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