University of Mazandaran

Iranian Biology Society

Purification and Characterization of Alginate Lyase from Mucoid *Pseudomonas aeruginosa* Strain 214

Hoda Gol Fakhrabadi, Parinaz Ghadam[*], Parisa Mohammadi and Ahya Abdi-Ali

Department of Biology, Faculty of Science, Alzahra University, Tehran, I.R. Iran

[*]Corresponding author: pghadam@alzahra.ac.ir

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that causes a variety of infections in compromised patients. The ability of *Pseudomonas aeruginosa* to produce chronic infection is based in part on its ability to biosynthesis of biofilm, and alginate is the major polysaccharide in the synthesized biofilm. So alginate degradation is very essential in the dispersion of *Pseudomonas aeruginosa* biofilm. Alginate lyase is an important enzyme in alginate degradation. This enzyme is different, especially with respect to molecular weight, pI and substrate specificity in various bacteria and even in various strains of a bacterium. The amount of alginate in mucoid strains is more than in nonmucoid strains. In this study, *P. aeruginosa* strain 214 was selected because it forms highly mucoidal colonies and thus it is a good candidate for alginate lyase preparation.
Alginate lyase was extracted from the periplasmic space of *P. aeruginosa* by the use of heat shock method. Thiobarbitoric acid assay was used for measuring the activity of alginate lyase. This enzyme showed the most activity in Tryptic Soy Broth (TSB) medium. The optimum concentration of sodium alginate was 0.02 mg/ml and the optimum activity of the enzyme was found in 20 min reaction time at 37°C. The enzyme was purified by a simple two-step procedure; ammonium sulfate precipitation and ion exchange column chromatography DEAE-Sepharose Cl-6B. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) suggested a molecular weight of approximately 40 kDa for alginate lyase.

Key words: Alginate; Alg lyase; Biofilm; *Pseudomonas aeruginosa*

Introduction

P. aeruginosa is an opportunistic pathogen that causes severe and life-treating infections in immunocompromised hosts such as patients with respiratory diseases, burns, cancers undergoing chemotherapy and cystic fibrosis (CF). Virulence factors produced by *P.aeruginosa* include numerous extracellular toxins, proteins and exopolysaccharides (Jain and Ohman, 2005). The most striking feature of *P.aeruginosa* strains is their highly mucoid phenotype, which is due to alginate overproduction (Eavns and Linker, 1973). *P.aeruginosa* alginate is composed of a linear polymer of β1-4 linked D-mannuronic acid and L-guluronic acid, which is variably, modified with O-acetyl groups on the mannuronic acid residues (Linhardt *et al*., 1986; Boyd and Chakrabarty, 1994; Rehm and Valla, 1997; Garron and Cygler, 2010), but in some articles, alginate of *P.aeruginosa* has

been reported to have just D-mannuronic acid (Wong *et al*., 2000; Xiao *et al*., 2006).

Alginates are enzymatically depolymerised by lyases (eliminases), which catalyze the cleavage of the glycosidic linkages by β-elimination with the formation of an unsaturated bond between the carbon atoms at the positions 4 and 5 of the uronic acid residue on the nonreducing end at the cleavage site (Boyd *et al*., 1993; Eftekhar and Schiller, 1994; Garron and Cygler, 2010). AlgL (or alginase) is produced by a number of bacteria including marine organisms (Sutherland and Keen, 1981; Doubet and Quatrano, 1982), Gram positive soil strain of *Bacillus circulans* (Hansen *et al*., 1984), Gram negative bacteria such as *Kelebsiella aerogenes* (Boyd and Turvey, 1977) and *P.aeruginosa* (Dunne and Buckmire, 1985; Eftekhar and Schiller, 1994). The presence of enzymes capable of degrading alginate has been described previously. The

first report of such an enzyme in *P.aeruginosa* strain infected with bacteriophage was by Bartell *et al*. (1966). Linker and Evans (1984), Dunne and Buckmire (1985) and Nguyen and Schiller (1989) have described polymannuronic acid depolymerases from mucoid *P.aeruginosa* strains. The enzymes described in these reports demonstrate some areas of similarity as well as major differences in the molecular weight and optimum pH of the enzymes, and also their ability for acetylated poly M alginate degradation. These findings suggest that more than one kind of alginate depolymerase may exist in *P.aeruginosa* strains (Nguyen and Schiller, 1989).

In this study, we purified the AlgL in a simple two-step process and characterized it from *P.aeruginosa* strain 214 that had been isolated from sputum.

Materials and Methods

Bacterial strain and culture conditions

Mucoid *P.aeruginosa* strain 214 (Abdi-Ali *et al*., 2006), originally isolated, was grown in Tryptic Soy Broth (TSB; Merck, Germany) and Nutrient Broth (NB; Merck) (Eftekhar and Schiller, 1994) overnight at 37°C with shaking (Wise Cube, USA). Biomass was collected by centrifugation (Centrifuge 5810 R , Germany) at 8000 g for 20 min at 4°C, washed twice with 1/10 volume of 0.03 M Tris-HCl buffer(Merck) , pH 7.5, containing 0.2 M $MgCl_2$ (Merck) (Schiller *et al*., 1993).

AlgL extraction procedure

A modification of heat shock method of Hoshino and Kangeyama was used for releasing of AlgL from the periplasmic space of *P. aeruginosa* (Hoshino and Kangeyama, 1980; Boyd *et al*., 1993; Schiller *et al*., 1993; Eftekhar and Schiller, 1994). Briefly, $MgCl_2$ (Merck) was added to the bacterial suspension at a final concentration of 0.2 M. Then the suspension was incubated at 37°C for 10 min followed by 15 min at 0°C (Hoshino and Kangeyama, 1980; Schiller *et al*., 1993). The bacterial cells were subjected to this alternating hot and cold temperature cycle 4 times before centrifugation at 8000 g, and the supernatant was stored at -20°C. Crude enzyme preparation from each procedure was then resuspended in 1/10 volume of 0.03 M Tris-HCl (Merck), pH 7.5, and tested for AlgL activity as described below.

Measurement of AlgL activity

The typical reaction mixture included 100 µl of the AlgL preparation, 100 µl of buffer [0.03 M Tris-HCl (Merck), pH 8.5, containing 9 mM $MgCl_2$ (Merck) and 0.5 M NaCl (Merck)] and 50 µl of sodium alginate (2 mg/ml) (Sigma Aldrich, England) as the substrate. The reaction mixture was incubated at 37°C for 20-30 min and the enzyme activity was measured by thiobarbituric acid (TBA; Sigma Aldrich) assay (Weissbach and Hurwitz, 1959; Schiller *et al*., 1993). The results were expressed in enzyme unit (EU), where one unit of enzyme activity was defined as the amount of enzyme that produced 1 nmol of β-formyl-pyruvate per min per ml at 37°C (10 nmol of β-formyl-pyruvate gives an Optical Density (OD) of 0.290 at 548 nm) (Nguyen and Schiller, 1989).In this study, the effect of different concentrations of substrate and the influence of reaction time on AlgL activity were surveyed.

Ammonium sulfate precipitation

The concentration of ammonium sulfate was adjusted by addition of solid ammonium sulfate (Merck) in 1.5 ml of the crude enzyme. Then the obtained solution was allowed to stand for 24 h at 4°C and the mixture was centrifuged at 15000 g for 15 min at 0 °C. The resultant precipitate was dissolved in 150 µl of 0.03 M Tris-HCl buffer (Merck), pH 7.8, and dialyzed (Sigma) overnight against 100 ml of the same buffer (Periss and Ashwell, 1962) and analyzed with SDS-PAGE.

DEAE-Sepharose Cl-6B

A DEAE-Sepharose Cl-6B column (Sigma) was prepared and equilibrated with 0.03 M Tris-HCl (Merck), pH 7.8. Then 50 µl of the above solution was loaded onto the column, washed with 0.03 M Tris-HCl (Merck), pH 7.8, containing 0.25 - 0.5 M NaCl (Merck), and the fractions were collected. Protein content and AlgL activity were determined for each fraction.

Poly acrylamide gel electrophoresis

A Laemmli SDS poly acrylamide gel (15% separating gel, pH 8.8 and 4% stacking gel, pH 6.8) was prepared. The enzyme preparations were diluted in 5X sample buffer containing 0.125 M Tris-HCl (Merck), pH 6.8, glycerol (Merck), beta-mercaptoethanol (Merck) and bromphenol blue (Merck) as the tracking dye. Then the samples were loaded into electrophoresis wells after boiling for 5 min. Electrophoresis (Power supply PST1002, Iran) was carried out at room temperature with 120 mV, until the tracking dye ran off the gel. The gel was stained with Coomassie Brilliant Blue R-250 (Merck) in 50% methanol (Merck) and 3.5% acetic acid (Merck) for 30 min, followed by destaining in 10% methanol (Merck) and 10 % acetic acid (Merck) overnight or until the gel background was cleared (Eftekhar and Schiller, 1994).

Results

The effect of different culture media on AlgL production

AlgL production by strain 214 was examined with TSB and NB. The enzyme was released from the bacterial suspension by the heat shock method and its lyase activity was measured with sodium alginate as the substrate with TBA assay at 548 nm. In TSB, the enzyme unit (EU) was greater (6.6 units) than in NB (5 units). Therefore, the strain 214 grown in TSB was used for the production of AlgL in all subsequent experiments.

The effect of different concentrations of substrate on AlgL activity

AlgL activity was increased by the sodium alginate concentration up to about 0.4 mg/ml (Fig. 1). Higher substrate concentration causes precipitation of the substrate and error in the assay results so 0. 2 mg/ml sodium alginate was chosen.

Influence of reaction time on enzyme activity

AlgL had the best activity initially with sodium alginate over the first 30 min (Fig. 2). Therefore, on the basis of this result, 20 min for incubation was optimum.
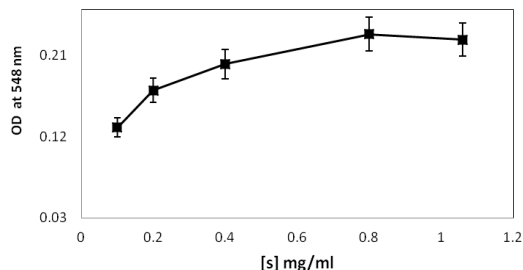

Fig. 1. The effect of different substrate concentrations in the enzymatic activity of AlgL. Reaction mixture containing a crude enzyme preparation from *P.aeruginosa* strain 214 and each of different substrate concentrations was incubated at 37°C and the enzyme activity was determined.
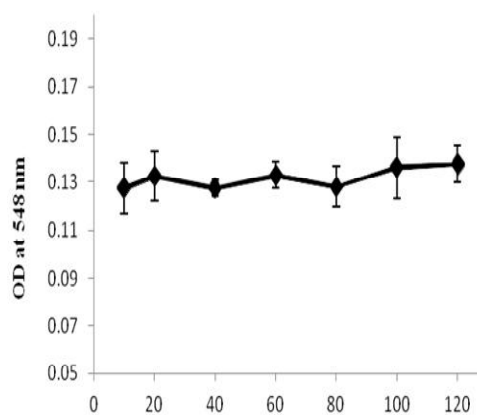

Fig. 2. Examination **Time (min)** :ic activity of AlgL at various periods of time of incubation. Reaction mixture containing a crude enzyme preparation from *P.aeruginosa* strain 214 and sodium alginate as substrate was incubated at 37°C and examined at different periods of time. Then the enzyme activity was determined.
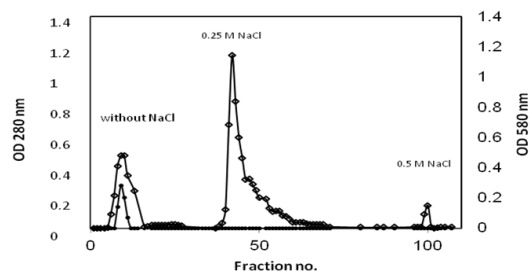

Fig. 3. Purification of AlgL with DEAE-Sepharose Cl-6B column chromatography. The protein was detected by measuring its absorbance at OD_{280} (◊), and AlgL activity was detected with TBA-reactive material OD_{548} (dotted line) after incubation for 20 min at 37°C.

Purification of AlgL

AlgL was released from periplasmic space of the clinically isolated mucoid *P.aeruginosa* strain 214 by the heat shock method.
The crude AlgL was precipitated with ammonium sulfate 70%, and was fractionated on DEAE-Sepharose Cl-6B column. The AlgL was eluted with buffer without NaCl (Fig. 3).
SDS-Poly Acryl amide Gel was used for purification quality analysis (Fig. 4). The major protein band was detected at approximately 40 KDa that TBA assay showed the AlgL activity.

Discussion

It is interesting that the same *P.aeruginosa* organism that produces a very viscous exopolysaccharide when grown in culture as well as *in vivo*, also produces an enzyme capable of degrading the above polymer. However, it should be considered that, in most cases, the native polysaccharide contains O-acetyl groups, which make it resistant to its own and other alginases (Linker and Evans, 1984). AlgL is an enzyme in alginate production and degradation pathways. Further purification and characterization of AlgL with a broad spectrum of activity against mucoid strains of *P.aeruginosa* are very essential for consideration of this enzyme as a possible therapeutic tool (May *et al*., 1991; Yamasaki *et al*., 2004; Jain and Ohman, 2005; Ramsey and Wozniak, 2005).
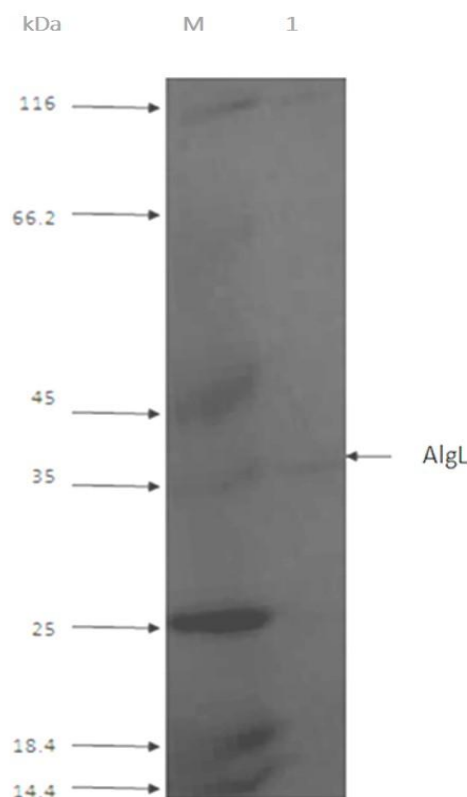

Fig. 4. The SDS-PAGE electrophoresis of AlgL from *P.aeruginosa* strain 214. Protein size marker (M), purified AlgL with DEAE-Sepharose Cl-6B column chromatography (1) were electrophoretically separated on a 15% running, 4% stacking gel and then stained.

Table 1. Purification of AlgL

Enzyme prep.	Alginate Lyase (EU/ml)	Protein (mg/ml)	Spec. Act. (EU/mg)	Purification fold
Crude	51.7	1.04	49.7	1
Prep. 1	103.4	0.65	159	3.2
Perp. 2	36.2	0.06	603.3	12.14

Perp. 1: After ammonium sulfate precipitation
Perp. 2: AlgL preparation eluted with buffer 0.03 M Tris-HCl, pH 7.8, from the DEAE-Sepharose Cl-6B

In this report, AlgL was extracted from the *P.aeruginosa* strain 214 originally isolated from a patient. This organism was shown to produce mucoid biofilm, so it is a good candidate for characterization of its AlgL because this enzyme is important in alginate production, and mucoid strains of *P.aeruginosa* have considerable amount of this enzyme.
AlgL is not usually secreted, but is either located in the periplasmic space or associated with the

outer cell membrane of *P.aeruginosa;* therefore, it can be released with the heat shock method (Eftekhar and Schiller, 1994). In this study, AlgL was simply purified in two steps by salting out and DEAE-Sepharose Cl-6B column chromatography. The results showed that this enzyme is not bound to this anion exchange column at pH 7.8. It shows that pI of this enzyme is greater than 7.8 and it is a basic protein so it is in contrast with the AlgL described by Eftekhar and Schiller (1994). Our results also showed the molecular weight of this AlgL is approximately 40 kDa, which is consistent with the other reports of AlgL (Schiller *et al.*, 1993; Boyd *et al.*, 1993; Eftekhar and Schiller, 1994; Rehm, 1998; Xiao *et al.,* 2006). Further investigation about the characterization of this enzyme might show whether it can degrade bacterial biofilm and has therapeutic importance.

Acknowledgment

References

Abdi-Ali A, Mohammadi-Mehr M, Agha Alaei Y. 2006. Bactericidal activity of various antibiotics against biofilm-producing *Pseudomonas aeruginosa*. *Int J Antimicrob Agent* 27: 196-200.

Bartell PF, Orr TE, Lam Gk. 1966. Polysaccharide depolymerases associated with bacteriophage infection. *J Bacteriol* 92: 56-62.

Boyd A, Chakrabarty AM. 1994. Role of AlgL cell detachment of *Pseudomonas aeruginosa*. *Appl Environ Microb* 60: 2355-2364.

Boyd A, Ghosh M, May TB, Shinabarger D, Keogh R, Chakrabarty AM. 1993. Sequence of the algL gene of *Pseudomonas aeruginosa* and purification of its AlgL product. *Gene* 131: 1-8.

Boyd J, Turvey JR. 1977. Isolation of a poly-alpha-L-guluronate lyase from *Klebsiella aerogenes*. *Carbohydr Res* 57: 163-171.

Doubet RS, Quatrano RS. 1982. Isolation of marine bacteria capable of producing specific lyases for Alginate degradation. *Appl Environ Microb* 44: 754-760.

Dunne WM, Buckmire FL. 1985. Partial purification and characterization of a polymannuronic acid depolymerase produced by a mucoid strain of *Pseudomonas aeruginosa* isolated from a patient with cystic fibrosis. *Appl Environ Microbiol* 50: 562-569.

Eavns LR, Linker A. 1973. Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. *J Bacteriol* 16: 915-921.

Eftekhar F, Schiller NL. 1994. Partial purification and characterization of a mannuronan-specific AlgL from *Pseudomonas aeruginosa*. *Curr Microbiol* 29: 37-42.

Garron M, Cygler M. 2010. Structural and mechanistic classification of uronic acid containing polysaccharide lyases. *Glycobiology* 20: 1547-1573.

Hansen JB, Doubet RS, Ram J. 1984. Alginase enzyme production by *Bacillus circulance*. *Appl Environ Microb* 47: 704-713.

Hoshino T, Kageyama M. 1980. Purification and properties of a binding protein for branched-chain amino acids in *Pseudomonas aeruginosa*. *J Bacteriol* 141: 1055-1063.

Jain S, Ohman D. 2005. Role of an AlgL for Alginate transport in mucoid *Pseudomonas aeruginosa*. *Infect Immun* 73: 6429-6436.

Linhardt R, Galliher P, Cooney C. 1986. Polysaccharide lyases. *Appl Biochem Biotechnol* 12: 135-145.

Linker A, Evans LR. 1984. Isolation and characterization of an alginase from mucoid strains of *Pseudomonas aeruginosa*. *J Bacteriol* 159: 958-964.

May TB, Shinabarger D, Maharaj R, Kato J, Chu L, DeVault JD, Roychoudhury S, Zielinski NA, Berry A, Rothmel RK. 1991. Alginate synthesis by *Pseudomonas aeruginosa*: A key pathogenic factor in chronic pulmonary infection of cystic fibrosis patients. *Clin Microbiol Rev* 4:191-206.

Nguyen LK, Schiller NL. 1989. Identification of a slime exopolysaccharide depolymerase in mucoid strains of *Pseudomonas aeruginosa*. *Curr Microbiol* 18: 323-332.

Periss J, Ashwell G. 1962. Alginic acid metabolism in bacteria. *J Biol Chem* 237: 309-316.

Ramsey DM, Wozniak DJ. 2005. Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infection in cystic fibrosis. *Mol Microbiol* 56: 309-322.

Rehm B. 1998. Alginate lysase from *Pseudomonas aeruginosa* CF1/M1 prefers the

hexameric oligomannuronate as substrate, *FEMS*. *Microbiol Lett* 165:175-180.

Rehm B, Valla S. 1997. Bacterial Alginate: Biosynthesis and applications. *Appl Microbiol Biot* 48: 281-289.

Schiller NL, Monday SR, Boyd CM, Keen NT, Ohman DE. 1993. Characterization of the *Pseudomonas aeruginosa* AlgL gene (algL): Cloning, sequencing, and expression in *Escherichia coli. J Bacteriol* 175: 4780-4789.

Sutherland IW, Keen GA. 1981. Alginases from *Beneckea pelagia* and *Pseudomonas* species. *J Appl Biochem* 3: 48-57.

Weissbach A, Hurwitz J. 1959. The formation of 2-keto-3 deoxyheptonic acid in extracts of *Escherichia coli B. J Biol Chem* 4: 705-714.

Wong TY, Preston LA, Schiller NL. 2000. AlgL: Review of major sources and enzyme characteristics, structure-function analysis, biological roles and applications. *Annu Rev Microbiol* 54: 289-340.

Xiao L, Han F, Yang Z, Lu X, Yu W. 2006. A novel AlgL with high activity on acetylated Alginate of *Pseudomonas aeruginosa* FRD1 from *Pseudomonas sp*. QD03. *World J Microbiol Biotechnol* 22: 81–89.

Yamasaki M, Moriwaki S, Miyake O, Hashimoto W, Murata K, Mikami B. 2004. Structure and function of hypothentical *Pseudomonas aeruginosa* protein PA1167 classified into family PL-7. *J Biol Chem* 279: 31863-31872.