

Purification and characterization of alginate lyase from mucoid *Pseudomonas aeruginosa* strain 214

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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. Thiobarbituric 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*

تخلیص و تعیین خصوصیت آلزینات لیاز سودوموناس آئروژینوزای موکوئیدی سویه ۲۱۴

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چکیده

سودوموناس آئروژینوزا پاتوژن فرصت طلبی است که در بیماران مستعد موجب عفونتهای متعددی می شود. توانایی سودوموناس آئروژینوزا در ایجاد عفونتهای مزمن به خاطر تولید بیوفیلم است که آلزینات پلی ساکارید مهم تشکیل دهنده آن می باشد. بنابراین تخریب آلزینات نقش مهمی در تخریب بیوفیلم بازی می کند. آلزینات لیاز آنزیم مهمی در تخریب آلزینات می باشد. وزن مولکولی، pI و ویژگی این آنزیم در باکتری های مختلف و حتی در سویه های یک باکتری متفاوت است. مقدار آلزینات در سویه های موکوئیدی بیشتر از غیرموکوئیدی ها می باشد. در این مطالعه سودوموناس آئروژینوزا سویه ۲۱۴ به دلیل تشکیل کلونی های بسیار موکوئیدی برای تهیه آلزینات لیاز انتخاب شد. آلزینات لیاز از فضای پری پلاسمیک سودوموناس آئروژینوزا با استفاده از شوک حرارتی استخراج و برای تعیین فعالیت آن از روش تیوباربتوریک اسید استفاده شد. این آنزیم بیشترین فعالیت را در محیط TSB نشان داد. غلظت بهینه سدیم آلزینات 0.02 mg/ml بود و فعالیت بهینه آن در ۲۰ دقیقه در ۳ درجه سانتی گراد مشاهده شد. با پروتوکل دو مرحله ای ترسیب با آمونیوم سولفات و کروماتوگرافی ستونی تعویض آنیونی DEAE-Sepharose Cl-6B آنزیم خالص گردید. با روش SDS-PAGE وزن مولکولی ۴۰ کیلو دالتون برای این آنزیم تخمین زده شد.

واژه های کلیدی: آلزینات؛ آلزینات لیاز؛ بیوفیلم؛ سودوموناس آئروژینوزا

Introduction

P. aeruginosa is an opportunistic pathogen that causes severe and life-threatening 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 & Ohman, 2005). The most striking feature of *P. aeruginosa* strains is their highly mucoid phenotype, which is due to alginate overproduction (Eavns & Linker, 1973). *P. aeruginosa* alginate is composed of a linear polymer of β 1-4 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 & Chakrabarty, 1994; Rehm & Valla, 1997; Garron & 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; Eftekhari & Schiller, 1994; Garron & Cygler, 2010). AlgL (or alginase) is produced by a number of bacteria including marine organisms (Sutherland & Keen, 1981; Doubet & Quatrano, 1982), Gram positive soil strain of *Bacillus circulans* (Hansen *et al.*, 1984), Gram negative bacteria such as *Kelebsiella aerogenes* (Boyd & Turvey, 1977) and *P. aeruginosa* (Dunne & Buckmire, 1985; Eftekhari & 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 & 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) (Eftekhari & 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 MgCl₂ (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 & Kangeyama, 1980; Boyd *et al.*, 1993; Schiller *et al.*, 1993; Eftekhari & Schiller, 1994). Briefly, MgCl₂ (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 & 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 μ l of the AlgL preparation, 100 μ l of buffer [0.03 M Tris-HCl (Merck), pH 8.5, containing 9 mM MgCl₂ (Merck) and 0.5 M NaCl (Merck)] and 50 μ l 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 & 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 & 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 μ l of 0.03 M Tris-HCl buffer (Merck), pH 7.8, and dialyzed (Sigma) overnight against 100 ml of the same buffer (Periss & 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 μ l 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 (Eftekhari & 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.

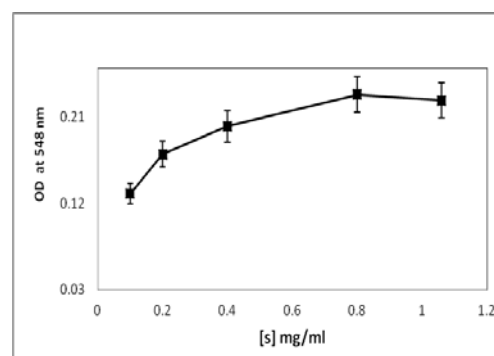


Figure 1. The effect of different substrate concentrations in the enzymatic activity of AlgL. Reaction mixture containing a crude enzyme preparation from *P.aeruginosa* strain 214 and each of different substrate concentrations was incubated at 37°C and the enzyme activity was determined.

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.

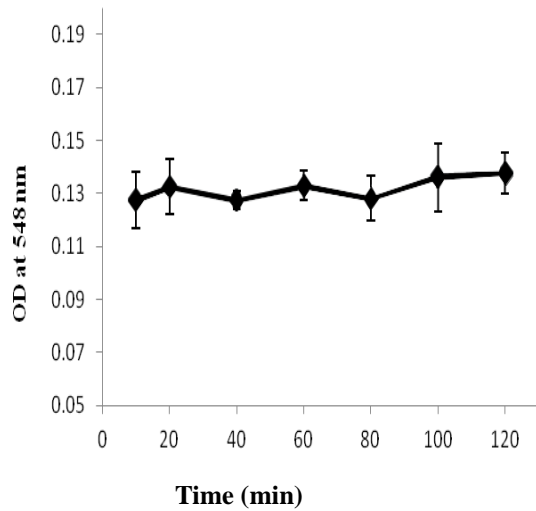


Figure 2. Examination of the enzymatic activity of AlgL at various periods of time of incubation. Reaction mixture containing a crude enzyme preparation from *P.aeruginosa* strain 214 and sodium alginate as substrate was incubated at 37°C and examined at different periods of time. Then the enzyme activity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

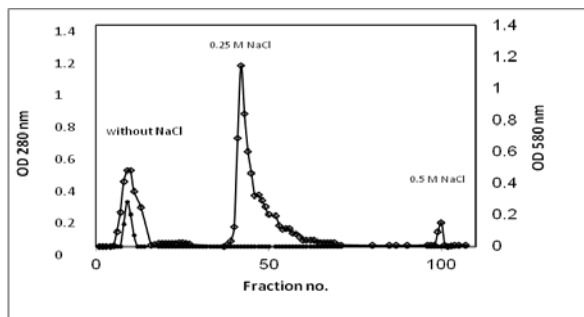


Figure 3. Purification of AlgL with DEAE-Sepharose Cl-6B column chromatography. The protein was detected by measuring its absorbance at OD₂₈₀ (◇), and AlgL activity was detected with TBA-reactive material OD₅₄₈ (dotted line) after incubation for 20 min at 37°C.

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.

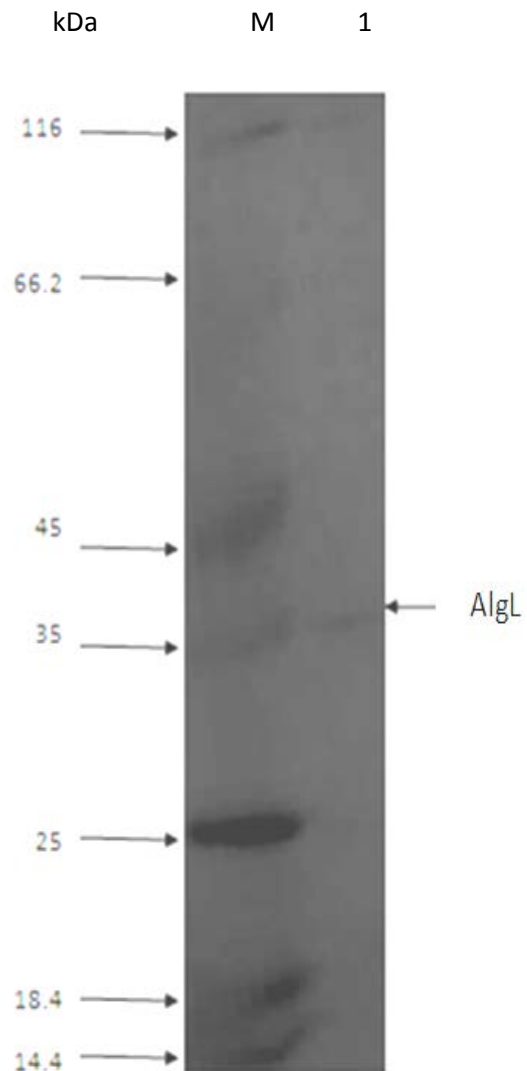


Figure 4. The SDS-PAGE electrophoresis of AlgL from *P.aeruginosa* strain 214. Protein size marker (M), purified AlgL with DEAE-Sepharose Cl-6B column chromatography (1) were electrophoretically separated on a 15% running, 4% stacking gel and then stained.

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
Perp. 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

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 & 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 & Ohman, 2005; Ramsey & Wozniak, 2005).

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 (Eftekhari & 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 Eftekhari

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; Eftekhari & 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

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