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Overproduction and properties of the mannuronate alginate lyase $AlxM_B$

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Abstract

In previous studies (Malissard et al., FEMS Microbiol. Lett. (1993) 110, 101-106), the alginate lyase AlxM of the marine bacterium ATCC 433367 was produced in *Escherichia coli* TC4/pAL-A3 with a yield of 50 μg per litre of culture. The polypeptide chain was cleaved between two cysteine residues, C169 and C183, themselves linked by a disulphide bridge. AlxM has now been overproduced in *E. coli* BL21(DE3)/pAL-Sur/pLysS. Under conditions in which formation of inclusion bodies can be avoided, the enzyme is synthesized as a catalytically active, water-soluble, unnicked polypeptide with a yield of 32 mg per litre of culture. It has been purified to protein homogeneity using a one-step procedure. The nicked $AlxM_A$ and unnicked $AlxM_B$ alginate lyases have identical alginate-degrading activities at high salt concentrations.

Keywords : Mannuronate alginate lyase ; Gene overexpression ; Inclusion bodies ; *Pseudomonas aeruginosa* alginate

1. Introduction

Alginates are linear uronic acid polymers in which β-D-mannuronate and α-L-guluronate residues are linked to form blocks of polymannuronate, polyguluronate and random sequences [1,2]. Alginates are synthesized as exopolysaccharides by seaweeds and bacteria. Bacterial alginates are mainly of the polymannuronate type with O-acetyl groups at the 2 and/or 3 position of D-mannuronate [3]. Alginate-producing *Pseudomonas aeruginosa* is a major pathogenic factor in cystic fibrosis patients [1]. Alginate lyases degrade alginates by a β-elimination mechanism [2,4].

Several alginate lyases are of known primary structure [5-10]. Among them, the alginate lyase $AlxM_A$ has been produced by *Escherichia coli* TC4 harbouring pAL-A3, a recombinant plasmid that carries the alginate lyase-encoding *alx*M gene from the marine bacterium ATCC 433367 [11]. $AlxM_A$ is a 265 amino acid residue protein that is nicked in the Cysl69-Cysl83 sequence (most likely between N177 and K178) [9]. The two polypeptides (of 20 kDa and 10 kDa, respectively) remain associated to each other by a disulphide bridge. They are freed under denaturing conditions.

As described below, a new recombinant plasmid has been constructed which allows *E. coli*, used as host, to overproduce the alginate lyase in a slightly modified, unnicked form, $AlxM_B$. The $AlxM_B$ enzyme has been purified and its properties have been compared to those of the nicked $AlxM_A$ enzyme.

2. Materials and methods

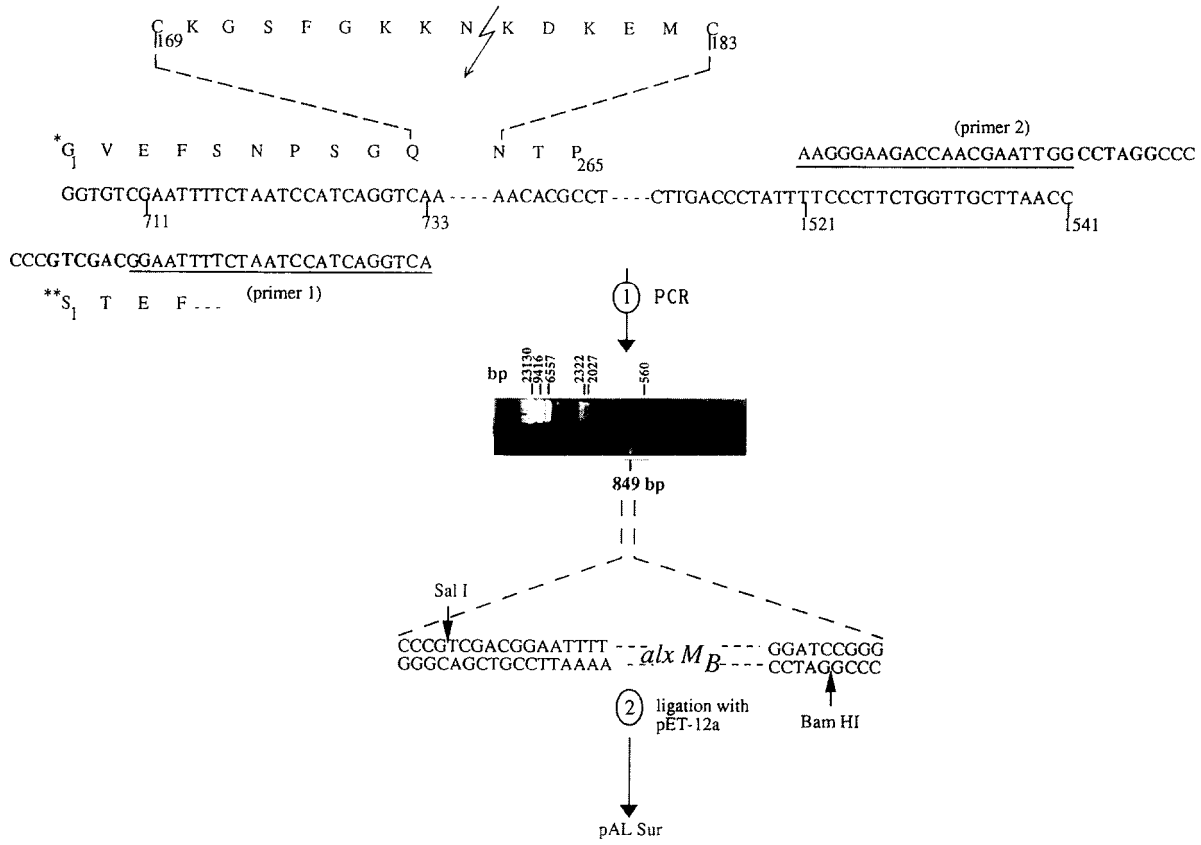
2.1. Bacterial strains and plasmids

E. coli BL2KDE3) and *E. coli* HMS174(DE3) (Novagen, Oxon, UK) were used as hosts. *E. coli* MV1190 was from Bio-Rad (Hercules, CA, USA). *E. coli* TC4, a spontaneous mutant of *E. coli* S17-1 [12] was a gift of Dr. L.O. Ingram (University of Florida). *E. coli* DH5α was from Life Technologies (Gaithesburg, MD, USA).

pAL-A4, a pUC20 recombinant possessing the 2088-3658 DNA segment of pAL-A3 [11], was prepared as described [9]. pET-12a (conferring ampicillin resistance), and plysS and plysE (conferring chloramphenicol

resistance) were from Novagen.

Fig. 1. *Construction of the alginate lyase $AlxM_B$-encoding pAL-Sur vector. The amino terminal dipeptide G1V of $AlxM_A$ (*) is replaced by the amino terminal dipeptide ST in AlxMB (* *). $AlxM_A$ is nicked between N177 and K178. For further details, see text.*



2.2. Cloning of $alxM_B$ in pET-12a. E. coli T12, T27 and T42

pET-12a had a *Sal*I site followed by a *Bam*HI site at the 3' end of a leader sequence. Corresponding restriction sites were created by PCR [13] at the ends of the *alx*M sequence encoding the mature $AlxM_A$ enzyme. The reaction was carried out using the Vent™ DNA polymerase (New England Biolabs, Beverly, MA, USA), pAL-A4 (≈ 100 ng) as template and the following oligonucleotides (Eurogentec, Belgium) as primers (Fig. 1). The 23 last bases of primer 1 were the 711-733 nucleotides encoding the peptide E3FSNPSG(Q) of the mature protein. The 21 last bases of primer 2 were complementary to the 1521-1541 nucleotides that occurred beyond the stop codon of $alxM_A$ [9]. PCR was carried out with a DNA Thermal Cycler (BiometraTrio Thermoblock) for 30 cycles, each with a 1 min denaturation at 94°C, a 1 min annealing at 46°C and a 150 s extension at 72°C. The cycles were prolonged by a 10 min incubation at 72°C.

The PCR product (purified on a Sephacryl S400 spun column; Pharmacia Biotech, Sweden) and pET-12a were digested with *Bam*HI and *Sal*I (for 2 h at 37°C) and ligated (at 4°C for 16 h and then at 7°C for 4 h). The resulting plasmid pAL-Sur (which encoded the alginate lyase AlxMB having the dipeptide ST of the amino terminus) was used to transform *E. coli* MV1190 following the conditions described in [14]. Transformants were selected on Luria-Bertani (LB) plates containing 50 μg of ampicillin per ml. Minipreparations of the supercoiled pAL-Sur were made. The pair pAL-Sur/pLysE was used to transform *E. coli* BL21(DE3) yielding *E. coli* T12 and *E. coli* HMS174(DE3) yielding *E. coli* T42. The pair pAL-Sur/pLysS was used to transform *E. coli* BL2KDE3) yielding *E. coli* T27.

2.3. λDE3 lysogenization

E. coli DH5α and *E. coli* TC4 were lysogenized with the Novagen kit designed for site specific integration of λDE3 (containing the T7 RNA polymerase-encoding gene) into the chromosome. The conditions used were those recommended by the manufacturer.

2.4. Enzyme assays

The substrates were the low viscosity, acetyl group-free *Macrocystis pyrifera* alginate (60% mannuronate; Sigma) and *Laminaria hyperborea* alginate (44% mannuronate; Algocean, Landernau, France) and a *P. aeruginosa* alginate (84% mannuronate; 46% acetyl groups) isolated from a cystic fibrosis patient [15]. Enzyme solutions containing 0.3% alginate were incubated at 37°C for 5 min in the indicated buffers. The unsaturated non-reducing groups produced by β-elimination were measured using 3-deoxy-D-manno-octulosonic acid (up to 50 nmol) as standard. The conditions described in [16] were modified as follows.

The incubation mixtures (0.4 ml) were successively: supplemented with 0.25 ml of 40 mM HIO_4 in 0.5 N H_2SO_4, buzzed, left to stand for 20 min at room temperature, supplemented with 0.25 ml of 2.6% $NaAsO_2$ in 0.5 N HCl, buzzed, supplemented (after the brown color had disappeared) with 0.5 ml of 0.6% thiobarbituric acid in water, buzzed, heated at 100°C for 15 min, supplemented (while still hot) with 1 ml dimethylsulfoxide, and left to cool to room temperature. The optical density was then read at 548 nm. One enzyme unit produced 1 μmol of non-reducing, unsaturated termini per min.

2.5. Purification of the $AlxM_B$ alginate lyase

E. coli cells from 1 l of culture grown under conditions allowing the enzyme to be synthesized in a water-soluble form, were collected by centrifugation, suspended in 125 ml of 200 mM sodium phosphate pH 7.0/50 mM NaCl, frozen and thawed (allowing the resident T7 lysozyme to perform cell lysis). DNAse I (EC 3.1.21.1, Sigma) was added at a final concentration of 1.7 U per ml. After 20 min at 20°C, the lysate containing 280 mg total protein (specific activity: 1.5 U/mg protein in 225 mM Tris-HCl, pH 7.5) was collected by centrifugation at 40000 × *g* for 20 min and loaded on a 2.5 × 8 cm hydroxyapatite column (Bio-Gel HTP, Bio-Rad) equilibrated against 200 mM sodium phosphate pH 7.0/50 mM NaCl. Inactive proteins were eliminated by washing the column with the buffer and the adsorbed alginate lyase was eluted at 350 mM sodium phosphate/50 mM NaCl using a linear gradient (10 bed volumes) of sodium phosphate pH 7.0, from 200 mM to 500 mM, in 50 mM NaCl (flow rate 120 ml h^{-1}). The active fractions (32 mg protein; specific activity: 12 U/mg in 225 mM Tris-HCl, pH 7.5) were concentrated by ultra-filtration on an Amicon YM-10 membrane and dialysed against 50 mM sodium phosphate pH 7.0/10 mM NaCl. The proteins were estimated as described [17] using bovine serum albumin as standard.

2.6. Cell fractionation

The periplasmic, membrane and cytoplasmic fractions of *E. coli* cells were prepared as described [18].

2.7. SDS-PAGE and amino acid sequencing

SDS-PAGE (15%) was carried out according to Laemmli and Favre [19]. Gels were stained with PAGE blue 83. Amino acid sequencing was performed with an Applied Biosystems 470 A amino acid microsequencer.

3. Results

Production of the unnicked $AlxM_B$ alginate lyase The transformants *E. coli* T12, T27 and T42 were obtained as described in the Materials and Methods section. Note that pAL-Sur contained *alx*M_B under the control of the promoter-operator *lac* and that transformation with pAL-Sur alone resulted in the elimination of the insert from the plasmid.

E. coli T12, T27 and T42 each were grown in LB medium containing 50 μg ampicillin and 30 μg chloramphenicol per ml at 37°C for 16 h. The cultures were diluted into fresh medium and growth was resumed under varying conditions of temperature, from 28°C to 37°C, and of isopropylthiogalactoside (IPTG) induction. IPTG, from 0.25 mM to 1 mM, was added to cultures of varying absorbances from 0.6 to 1.0 at 600 nm, and the induced cultures were maintained at the selected temperature for 1-20 h. *E. coli* T12, T27 and T42 produced the alginate lyase in an active, water-soluble form only at temperature below 30°C (Fig. 2). At temperatures higher

than 30°C, the protein underwent aggregation into inclusion bodies.

The optimal conditions for enzyme production were to grow the best producer, i.e. *E. coli* T27, at 28°C, to add 0-4 mM IPTG to mid-log phase cultures and to continue growth for 5 h at 28°C. The water-soluble enzyme was purified to protein homogeneity using the one-step procedure described in Materials and Methods (Fig. 3). The yield of the purification was 90%. The amplification factor was 640, compared to the production obtained with *E. coli* TC4/pAL-A3 [9].

3.1. Properties of the unnicked $AlxM_B$ alginate lyase

As expected from the genetic construction, the enzyme had the amino terminal sequence STEFSNP. Serine was the only terminal amino acid residue and the enzyme was not cleaved under denaturing conditions.

At low ionic strength (in 225 mM Tris-HCl, pH 7.5) and by reference to the *M. pyrifera* alginate, the unnicked $AlxM_B$ enzyme had a two-fold decreased specific activity on *L. hyperborea* alginate (7 U/mg protein) and a two-fold increased specific activity on *P. aeruginosa* alginate (25 U/mg protein). The nicked $AlxM_A$ enzyme had a similar specificity profile [9]. The activity of both $AlxM_B$ and $AlxM_A$ enzymes (on *M. pyrifera* alginate) increased linearly as the ionic strength of the reaction mixture increased (Fig. 4). At ionic strengths above 1 M, both enzyme forms had maximal and comparable activity ≈ 80-90 U/mg protein. Increasing ionic strengths could be obtained by increasing the phosphate molarity or by supplementing a phosphate buffer of low molarity with increasing NaCl concentrations. The AlxMB enzyme underwent 50% denaturation after a 10 min incubation at 45°C.

As an attempt to nick $AlxM_B$ into $AlxM_A$ in vitro, $AlxM_B$ was incubated (in 50 mM sodium phosphate, pH 7.0) with samples of the cytoplasmic, membrane and periplasmic fractions isolated from *E. coli* TC4/pAL-A3 (i.e. the $AlxM_A$ producer). Nicking activity was found to be associated with the membrane fraction only. Nicking resulted in an increased enzyme activity at low ionic strength and the nicked enzyme gave rise to the 20 kDa and 10 kDa fragments under reducing conditions. Prolonged incubations with the membrane fraction, however, resulted in a more complete degradation of the protein.

As an attempt to produce the nicked $AlxM_B$ in vivo, *E. coli* DH5*α* and *E. coli* TC4 each were lysogenized (see the Materials and Methods section) and co-transformed with pAL-Sur and pLysS. Both transformants, grown under the same conditions as those described for *E. coli* T27, synthesize the active enzyme in the unnicked form. The lack of nicking activity might be attributed to the much reduced rate of synthesis of the outer membrane protease OmpT in *E. coli* cells grown at a temperature below 32°C.

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Fig. 2. *Effect of growth temperature on the production of the alginate lyase $AlxM_B$ by E. coli T27. Cultures were grown at (■) 28°C; (○) 30°C; (□) 32°C; and (●) 37°C. The induction was done at $A_{600\ nm}$ = 0.6, with 1 mM IPTG. Aliquots of 3.5 ml were removed at different times after induction and the crude enzyme solutions (500 µl) were prepared by freezing and thawing the cells. The enzyme activity was measured on 0.3% M. pyrifera alginate in 225 mM Tris-HCl buffer, pH 7.5.*

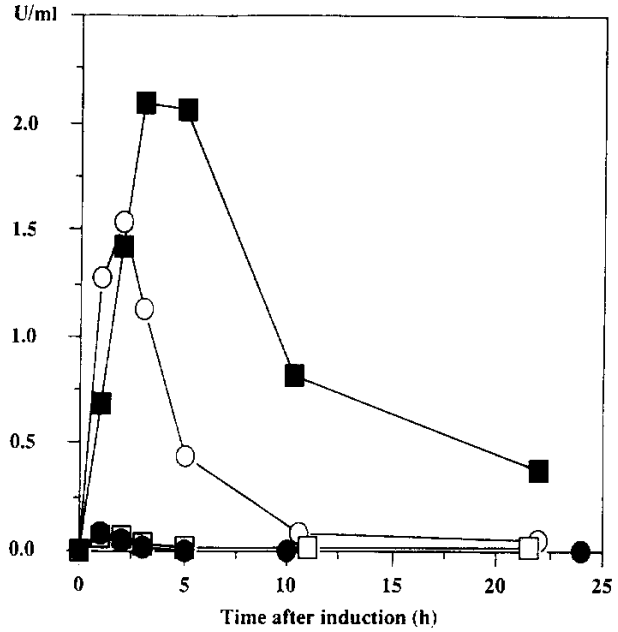


Fig. 3. *Purification of the alginate lyase $AlxM_B$ by hydroxyapatite chromatography and SDS-PAGE analysis. For conditions, see text. Flow rate: 2 ml per min. Sample fractions: 6 ml. The enzyme activity was measured on 0.3% M. pyrifera alginate in 225 mM Tris-HCl pH 7.5. □ : absorbance at 280 run; ●: enzyme unit per ml; dashed line: sodium phosphate concentration gradient. Insert (SDS-PAGE, 15%) of crude lysate preparation (lane 1); proteins not retained on the column (lane 2); $AlxM_B$ enzyme (lane 3). Proteins per sample : 10 µg. Proteins of standard molecular masses are shown on the left lane.*

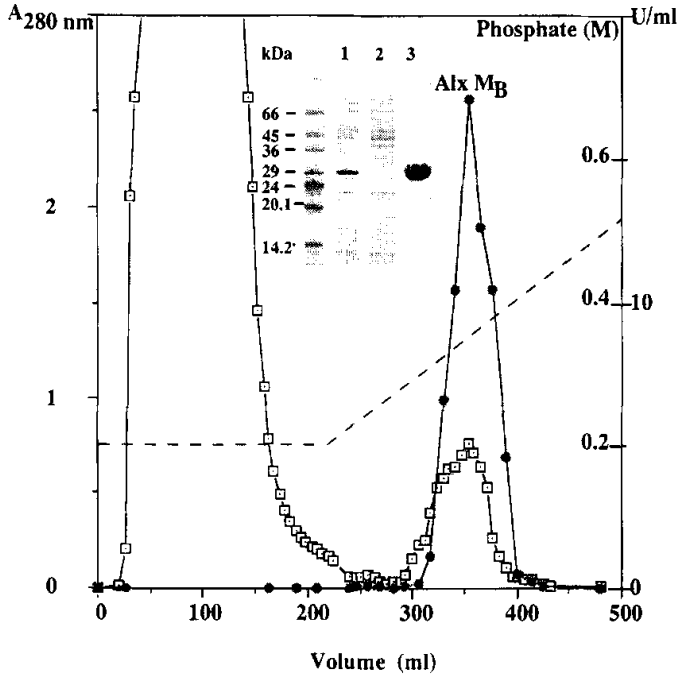
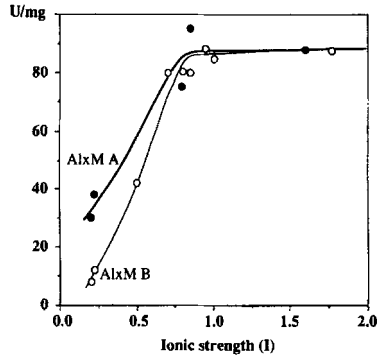


Fig. 4. *Effect of increasing ionic strength on the specific activity of the alginate lyases $AlxM_B$ (○) and $AlxM_A$ (●) at pH 7.5. The M. pyrifera alginate (0.3%) was used as substrate. Buffers used: 225 mM Tris-HCl (I = 0.22) · 37 mM sodium phosphate (I = 0.2); + 300 mM NaCl (I = 0.5); + 600 mM NaCl (I = 0.8); + 750 mM NaCl (I = 0.95) · 75 mM sodium phosphate + 300 mM NaCl (I = 0.7); + 450 mM NaCl (I = 0.85); + 600 mM NaCl (I = 1) · 300 mM sodium phosphate (I = 1.6); + 150 mM NaCl (I = 1.75).*



4. Discussion

Large amounts of alginate lyases are necessary to allow the β-elimination mechanism of the catalysed reaction to be studied, the three-dimensional structures of the enzymes to be elucidated and their possible use in the treatment of *P. aeruginosa*-infected cystic fibrosis patients to be evaluated. For that purpose, alginate lyases of different origins and without obvious sequence similarities, have been overproduced in *E. coli* [10] and *Bacillus subtilis* [20].

In previous work [9], the alginate lyase $AlxM_A$ encoded by a gene of the marine bacterium ATCC 433367 had been produced in the periplasm of *E. coli* TC4/pAL-A3 with a yield of about 50 μg per 1 of culture. Overproduction of the enzyme in a slightly modified form, $AlxM_B$, has now been achieved in *E. coli* BL21(DE3)/pAL-Sur/pLysS. Under optimal growth conditions, $AlxM_B$ is synthesized in an enzymatically active, water-soluble form with a yield of 32 mg per 1 of culture.

$AlxM_B$ differs from $AlxM_A$ by the presence of the dipeptide ST instead of the dipeptide GV at the amino terminus of the protein and by the occurrence of an intact (unnicked) C169-C183 peptide segment (see Introduction). It is known that the activation of many bacterial toxins occurs by proteolytic cleavage at a defined site, often followed by reduction of a disulfide bond, thereby freeing the catalytic domain [21]. Such an activation process does not apply to the AlxM alginate lyases. The nicked $AlxM_A$ enzyme and the unnicked $AlxM_B$ enzyme have similar specificity profile and alginate-degrading activity at high ionic strength. High salt concentrations probably confer optimal conformation to the active site, as expected for an enzyme originating from a marine bacterium.

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