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Émilie Gillon, Annabelle Varrot, Anne Imberty. LecB, a High Affinity Soluble Fucose-Binding Lectin from *Pseudomonas aeruginosa*. *Methods in Molecular Biology*, 2020, Lectin purification and analysis (2132), pp.475-482. 10.1007/978-1-0716-0430-4\_46 . hal-02554312

**HAL Id: hal-02554312**

**<https://hal.science/hal-02554312v1>**

Submitted on 25 Apr 2020

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# LecB, a High Affinity Soluble Fucose-Binding Lectin from *Pseudomonas aeruginosa*

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**Running Head:** LecB/PA-III from *Pseudomonas aeruginosa*

**Key words:** *Pseudomonas aeruginosa*, soluble lectin, fucose, blood-group oligosaccharide, microcalorimetry,

## Abstract

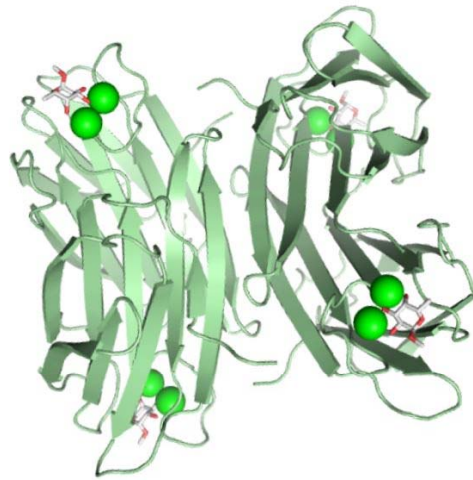
LecB/PA-III (Pfam PF07472) from bacterium *Pseudomonas aeruginosa* is a fucose-binding lectin with unusual high affinity for glycans. The occurrence of LecB and related proteins is limited to few opportunistic bacterial species, some of them being responsible for severe infections in immune compromised patients. This lectin is therefore of interest as a target for the design of anti-infectious compounds, but can also be used for research and biotechnology. LecB is a small protein that can be produced in good quantity in recombinant system and purified by affinity chromatography.

## 1 Introduction

*Pseudomonas aeruginosa* is one of the main human opportunistic pathogens and is responsible of infections such as folliculitis, keratitis, otitis but also pneumopathies in cystic fibrosis patients resulting in morbidity and mortality [1, 2]. Among several virulence factors, *P. aeruginosa* produces two soluble lectins named LecA (or PA-IL) and LecB (or PA-III) that are specific for galactose and fucose, respectively [3]. LecB consists of four subunits of 11.73 kDa, each with a high affinity binding site for L-fucose and fucose-containing glycoconjugates [4]. The lectin also binds to D-mannose but with lower affinity. LecB is produced in the cytoplasm of *P. aeruginosa* but has been shown to be located in the outer membrane and involved in biofilm formation [5].

The crystal structure of LecB in complex with fucose [6] and with mannose [7] uncovered the molecular basis of its ligand recognition. LecB forms homotetramer, each monomer presenting a fucose binding site with two calcium ions that are directly involved in carbohydrate coordination. LecB displays exceptionally high affinities for fucose, with  $K_d$  values in the micromolar range [8]. It binds to a large range of fucosylated antigens (ABH, Lewis, P and I systems) present on human tissues and in human milk [9, 10] with highest reported affinity in the nM range for Lewis a epitope [9], and for biantennary *N*-glycans presenting two H-type epitopes at the extremities of branches [11]. LecB is a target of interest for anti-adhesion and anti-biofilm compounds and the search for glyco-derived drugs with potential therapeutical application is very active [12, 13].

As other lectins with specificity towards human glycans, LecB is also of biotechnical interest for the purification of glycoproteins or the labelling of glycoconjugates on tissues. LecB is now included in the few lectin-arrays proposed in different laboratories for the fine characterization of glycans. Due to its propensity to easily form crystals, LecB has been utilized as a template for structural determination of smaller biomolecules that are difficult to crystallize by themselves : after attachment of a fucose residue, modified DNA duplexes or short anti-bacterial peptides could be co-crystallized with LecB and structurally characterized [14, 15].



LecB <sub>PA01</sub>	1	ATQGVFTLPANTRFGVTAFANSSGTQTVNVLVNNETAATFSGQSTNNAVIGTQVLNS	:	57
LecB <sub>PA7</sub>	1	ATQGVFTLPANTRFGVTAFANSSATQTVKVLVNNETAATFTGQSTNNAVIGSQVLNS	:	57
LecB <sub>PA14</sub>	1	ATQGVFTLPANTQFGVTAFANSSAGTQTVNVQVNNETVATFTGQSTNNAIGSKVLNS	:	57
LecB <sub>PA01</sub>	58	GSSGKVQVQVSVNGRPSDLVSAQVILTNELNFALVGSEDDTDNDYNDVAVVINWPLG	:	114
LecB <sub>PA7</sub>	58	GGGGKVQIQVSVNGRPSDLVSAQVILANELNFALVGSEDDTDNDYNDVAVVINWPLG	:	114
LecB <sub>PA14</sub>	58	GGGGKVQILVSVNGRPSDLVSAQVILANELNFALVGSEDDTDNDYNDVAVVINWPLG	:	114

**Figure 1:** Alignment of amino acid sequences for LecB sequences and crystal structure of LecB<sub>PA14</sub> in complex with  $\alpha$ -methyl-fucoside (5A6X). Protein is represented by ribbon, monosaccharides by sticks and calcium ions by spheres.

Most studies have been performed using LecB from *P. aeruginosa* PAO1 strain. However, recent works demonstrated sequence variations in LecB when analyzing the many isolates from clinical or environmental origin. Three different clusters of sequences were identified, represented by LecB from PAO1, PA7 and PA14 virulent strains [11, 16]. Some of the amino acid variations are located near the fucose binding site but affect only slightly the specificity and affinity of LecB. LecB-like lectins are also present in a limited number of bacteria from the *Ralstonia*, *Chromobacterium* and *Burkholderia* species. CV-III from *Chromobacterium violaceum* is very similar to LecB [17], while RS2L from *Ralstonia solanacearum* is specific for mannoside [18]. Such variation in specificity has been demonstrated to be due to one amino acid variation at position 22 [19]. *Burkholderia cenocepacia* hosts four different LecB-like proteins with dimeric association of lectin and extension by several other proteins [20, 21]. The details of production and characterization listed here can be therefore applied, with small adjustments to the whole family of LecB-like lectins.

## 2 Materials

Prepare all solutions using analytical grade reagents and ultrapure water and then filter on 0.22  $\mu$ M membrane. Follow waste disposal regulations.

### 2.1 LecB Production

1. Dry block heater at 42 °C.
2. Incubation shaker with cooling option.
3. 250 mL and 3 L baffled Erlenmeyer flasks.
4. Competent bacteria : *Escherichia coli* BL21(DE3)

5. Plasmid: pET25b-LecB
6. Lennox L Broth Base.
7. 100 mg/mL ampicilline.
8. 1 M Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG).
9. 1 L polycarbonate centrifuge bottle.
10. 50 mL sterile conical centrifuge tubes.
11. Centrifuge with adapted rotors.

## 2.2 *LecB Purification*

1. Endonuclease such as Benzonase or Denarase at 250 units/ $\mu$ L .
2. Rotating wheel.
3. One shot table top cell disruptor (Constant systems Ltd).
4. 35 mL centrifuge tube and centrifuge wth rotor going to 24,000  $\times g$
5. 0.45  $\mu$ m PES syringe filter.
6. C10/10 column with flow adapter (GE Healthcare Life Sciences).
7. Equilibration buffer : 20 mM Tris pH 7.5 100 mM NaCl and 100  $\mu$ M CaCl<sub>2</sub> (*see Note 1*).
8. Elution buffer : 20 mM Tris pH 7.5 100 mM NaCl and 100 mM D-Mannose.
9. Low or Medium-Pressure Chromatography Systems such as Akta Prime (GE Healthcare Life Sci) or NGC (Bio-Rad).
10. 5mL collection tubes.
11. Snakeskin dialysis tubing 3500 MWCO (Thermo Fisher Scientific).
12. 20 % ethanol

## 2.3 *Quality Control by Evaluation of Affinity*

1. ITC200 Microcalorimeter (Malvern Panalytical).
2. Nanodrop 2000 Spectrophotometer (Thermo Scientific).
3. ITC buffer : 20 mM Tris pH 7.5 100 mM NaCl and 100  $\mu$ M CaCl<sub>2</sub>.
4.  $\alpha$ -methyl fucopyranoside (SIGMA 32198).

## 3 **Methods**

### 3.1 *LecB Production*

#### 3.1.1 **Transformation and Preculture**

1. The plamid pET25b-LecB has been obtained from template DNA from *Pseudomonas aeruginosa* ATCC 33347 as previously described [8] and is available upon request.
2. Add 1  $\mu$ L of pET25b-LecB plasmid to 100  $\mu$ L of competent BL21(DE3) cells before 10 mn incubation on ice.
3. Heat shock at 42  $^{\circ}$ C for 45 s before a 3 min stay on ice.
4. Add 900  $\mu$ L of liquid LB and incubate horizontally for 1 hr at 37  $^{\circ}$ C with shaking at 180 rpm.
5. Spread 100  $\mu$ L on a petri dish with LB-agar and 100  $\mu$ g/mL ampicilline and incubate at 37  $^{\circ}$ C overnight.
6. Transfer 1 colony to 10 mL liquid LB containing 100  $\mu$ g/mL ampicilline and incubate overnight with shaking at 180 rpm.

#### 3.1.2 **Culture**

1. Inoculate 1 L of liquid LB supplemented with 100  $\mu$ g/mL ampicilline in 3 liters Erlenmeyer with the overnigth preculture.
2. Incubate at 37  $^{\circ}$ C with shaking at 180 rpm until OD<sub>600nm</sub> reached 0.6.
3. Switch the temperature to 30  $^{\circ}$ C, induce expression by adding 0.25 mM IPTG and culture for 3 hours at 180 rpm.

4. Centrifuge at  $6,000 \times g$ ,  $4^\circ\text{C}$  for 15 min.
5. Resuspend the pellet in 35 mL liquid LB, transfer in 50 mL sterile conical tube and centrifuge  $6,000 \times g$  for 10 min.
6. Remove supernatant and store the pellet at  $-20^\circ\text{C}$  until use.

### 3.2 *LecB Purification*

1. Resuspend the pellet in 30 mL of equilibration buffer.
2. Break the cells using a one shot table top cell disruptor at a pressure of 1.9 kbar.
3. Centrifuge  $24,000 \times g$ , 30 min at  $4^\circ\text{C}$  to clarify the sample.
4. Filter the supernatant on  $0.45 \mu\text{m}$  with syringe filter.
5. Equilibrate a 10 mL D-Mannose agarose resin packed in C10/10 column connected to automated chromatography system such as NGC system with 5 column volumes (CV) of equilibration buffer at a flowrate of 1 mL/min.
6. Load the filtered supernatant at a flow rate of 1 mL/min and collect the flowthrough.
7. Rinse the column with the equilibration buffer to wash out contaminants until the baseline of  $\text{OD}_{280\text{nm}}$  on the chromatogram stabilized to a low value (3 mL/min flowrate).
8. Elute the protein with the elution buffer using a flowrate of 3 mL/min and collect fraction of 5 mL.
9. Rinse the column with 3 CVs of ultrapure water at a flowrate of 3 mL/min and 2 CVs of 20 % ethanol at flowrate of 1 mL/min prior storage at  $4^\circ\text{C}$ .
10. Collect the fractions of the eluted peak and dialyzed against ultrapure water for 1 week, changing the bath each morning and evening.
11. Lyophilize protein and store at  $-20^\circ\text{C}$  (*see Note 2*).

### 3.3 *Quality Control by Evaluation of Affinity*

Isothermal titration calorimetry (ITC) is an analytical technique, which is considered as the gold standard for analyzing intermolecular interactions in solution. It is widely used for quality control in biotechnology since it gives access to quantitative analysis of the interaction of a protein with its ligand. A single experiment allows to determine stoichiometry ( $n$ ), binding enthalpy ( $\Delta H$ ), and affinity (i.e. dissociation constant,  $K_d$ ). Since the method is based on the heat released or absorbed in the binding process, it is very well suited for protein carbohydrate interaction, where the large number of hydrogen bonds result in strong exothermic signal [22].

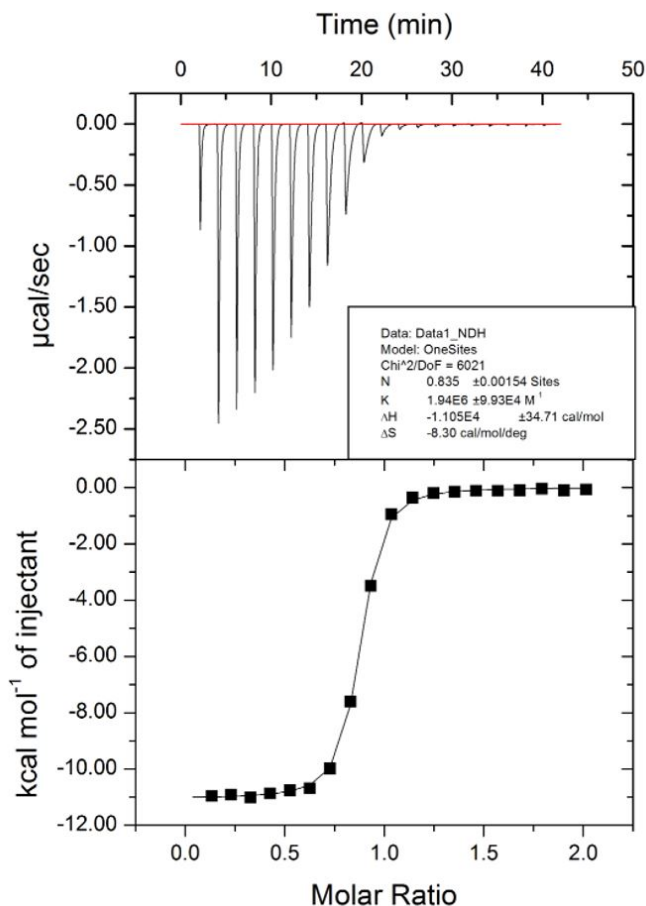
#### 3.3.1 *Sample Preparation :*

1. Prepare  $400 \mu\text{L}$  of LecB at  $50 \mu\text{M}$  in ITC buffer from the lyophilized protein. Weight  $1.5 \times$  excess amount of the necessary protein, dissolve in the corresponding volume of buffer, centrifuge  $7,000 \times g$  for 5 min.
2. Recover the supernatant and determine the precise concentration by spectroscopy at 280 nm using a molecular weight of 11,732 daltons per monomer and a theoretical molar extinction coefficient of 6,990 corresponding to Absorbance 0.1 % (= 1 g /L) of 0.596.
3. Adjust the concentration to  $50 \mu\text{M}$  with the ITC buffer.
4. Prepare  $70 \mu\text{L}$  of 0.5 mM of  $\alpha$ -methyl fucoside ( $\alpha\text{MeFuc}$ ) in the same buffer as the lectin (*see Note 3*).
5. Degas all samples.

#### 3.3.2 *ITC Experiment*

1. Fill the measuring cell with  $50 \mu\text{M}$  LecB solution and the syringe with 0.5 mM  $\alpha\text{MeFuc}$  solution. Cell volume is  $200 \mu\text{L}$  and syringe volume is  $40 \mu\text{L}$ .
2. Setup the parameters to DP 8, injection number 20, injection volume of  $1 \mu\text{L}$  for the first one and  $2 \mu\text{L}$  for all following ones with an injection delay of 120 s.

- Use the Origin software of the microcalorimeter for data analysis (*see Note 4*). The default parameters with a “one-site” model is fully compatible with the present experiments. Data will be obtained as indicated in Fig. 2 when clicking on the “ITC data” button (*see Note 5*).



**Figure 2:** Typical ITC experiment of titration of LecB<sub>PAO1</sub> by α-methyl-L-fucoside with thermogram (top) and integrated data (bottom). The observed stoichiometry of 0.835 indicates here that 16.5% of the protein is inactive. This does not affect the determination of thermodynamic constant, since they are expressed as a function of the concentration of ligand. The enthalpy of binding is -11.05 Kcal/mol (- 46kJ/mole) with a weak unfavorable contribution of entropy ΔS of -8.3 cal/mol/deg (ie TΔS = -10.3 kJ/mol) which is in full agreement with previous data [23].

- Quality control is performed by comparing the obtained thermodynamic of binding with literature [23]. LecB was reported to have a submicromolar affinity for aMeFuc ( $K_d = 430$  nM) with strong enthalpy ( $\Delta H = -41.3$  kJ/mol) and a stoichiometry corresponding to approximately one binding site per monomer ( $n = 0.77$ ).

#### 4 Notes

1. LecB is a calcium-dependent lectin and it is advised to maintain a CaCl<sub>2</sub> concentration of 100 μM through the purification process. If needed, this concentration can be decreased to 10 μM.
2. LecB is a very robust protein that can be lyophilized and stored at -20 °C for months or years. It is only recommended to aliquot in small quantities to avoid repeating cycles of defreezing and refreezing.
3. For a perfect ITC measurement, the main recommendation is to avoid buffer mismatch. Use exactly the same buffer for the protein solution and the sugar solution.
4. Alternatives to Origin are available in the public domain. The suite of integrated software packages NITPIC, SEDPHAT and GUSSE offer powerful treatment of data with automated shape analysis of the injection peaks [24]. It also allows for integrating results from different calorimetric titration experiments into a global analysis
5. In the Origin software, the default units are calories. This could be change to international units Watt and Joules using the Tab ITC and then Display Watt/Joules.

#### Acknowledgements

The authors acknowledge support by the ANR PIA Glyco@Alps (ANR-15-IDEX-02), Labex ARCANÉ and CBH-EUR-GS (ANR-17-EURE-0003), and the French Cystic fibrosis association Vaincre la Mucoviscidose.

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