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Partial purification of type A and B flagellin from *Pseudomonas aeruginosa* for preclinical use

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***Abstract*---**Background: Due to their importance in vaccination, we tried in this study to purify the flagellin type a and type b separately from *P. aeruginosa* by a simple and cheap method with acceptable purity for preclinical use. Methods: Specific primer was used to detect the type a and b flagellin gene (*fliC* gene) in *P. aeruginosa* clinical isolates. Flagellin was purified from *P. aeruginosa* according to standard procedure with some modifications. Protein concentration and protein absorption were measured using a special spectrophotometer at 270 wavelength. Endotoxin removal was done using a special column. Then, the presence of flagellin was detected by SDS-PAGE. Results: The results showed two types of flagllin genes in different strains; type a (1020 bp) and type b (1250 pb) that amplified by PCR. Results also showed an appropriate concentration of purified protein by the used method with acceptable purity and decreasing the endotoxin to an acceptable level for preclinical use (less than 2.2 EU/ml). Conclusion: This research focused on the isolation and purification of flagellin protein from *P. aeruginosa*. This study showed that the method used to isolate flagellin protein was highly acceptable, considered cost-effective, few steps required, few equipment with acceptable purity, and decreased endotoxin (LPS) for preclinical use.

Keywords*---Pseudomonas aeruginosa*, Flagellin, Purification, Endotoxin Removal, Endotoxin detection, *fliC* gene.

Introduction

Pseudomonas aeruginosa remains one of the most important pathogens in nosocomial infections, with high related morbidity and mortality (1). *P. aeruginosa* have unipolar flagellum that polymerized from monomer protein called flagellin that attach to the trans-membrane motor complex (2). There are two types of flagellin identified in this bacteria called type a and type b flagellin that can be recognized on a molecular level. There is no phase variation between the two types; and there is no class switching between them in a single strain (3). This structure play an important role in the host immune response to this bacteria by interaction with immune cells and non-immune cells in addition to its basic function (bacterial motility).

The flagellin activate host cell signaling pathways by two way: first, its working as pathogen associated molecular patterns (PAMPs) that interact with immune response by binding with extracellular Toll like receptor 5 (TLR5) that activate the pro-inflammatory immune response. TLR5 mediate the immune response against *P. aeruginosa* in the epithelial cells of lung by enhancing neutrophil recruitment (4–6), and participate in stimulating monocyte and macrophage to produce pro-IL-1β (7). Second, by its promoting the inducing of maturation IL-1β by the activation of NAIP-NLRC4-inflamosome after its translocated to the mammalian cells cytoplasm through the type 3 secretion system (T3SS) (8,9). Notably, IL-1ß have an paracrine and autocrine effects through which it promote the phagocytosis (7,10). Also in last five years many studies show that its inducing Th1 and Th17 immune response in lung and systemically (11–13).

Although most studies of *P. aeruginosa* therapeutics focus on antibiotics currently in use or in the pipeline, few authors developing translational strategies aimed at using virulence factor antagonists as adjuvant therapies. *P. aeruginosa* contain two types of flagellin proteins only (type a and b), and they differentiate by molecular sizes. There are no any switching between the two types and there are no any phase variation in the same strain (14). Many studies showed their importance in vaccination such as Faezi and his colleagues (15) who showed that the active immunization of mice by flagellin type A made it protected from burn infection.

In connection with the above, and due to their importance as a candidate in vaccination, this study focused on the purification of two flagellin types from *P. aeruginosa* by using simple, cheap, and most available method with acceptable purity suitable to that purpose.

Materials and Methods

Isolation of Bacterial Strains

Pseudomonas aeruginosa strains were isolated from the main hospital in Al-Najaf province (Al-Sadr medical city). The Identification of bacterial isolate was achieved a classical identification methods according to Forbes *et al.* (16) and confirmed its identification using VITEK 2 system (Biomerieux/ France). Verbal and writing consents were taken from each patient before sampling. The protocol of study, the

subject information, and consent form were reviewed and approved by a local committee of ethics (Najaf Health directorate, Iraq).

Detection the type of flagellin *fliC* gene

Specific primer was used to detect the type a or b flagellin gene (*fliC* gene) in the isolated bacteria. The primer sequence and PCR condition was depend on the article of Ertugrul *et al* (3). A specific primer to the N-terminal "CW46" and to the C-terminal "CW45" that consider conserved regions in the flagellin gene of *P. aeruginosa* that used in PCR to amplify it (3), the forward primer is "CW45-F: 5'-GGCAGCTGGTTNGCCTG-3'" and reverse primer is "CW45-R: 5'-GGCCTGCAGATCNCCAA-3'", that give two bands; 1020 bp for type a and 1250 bp for type b; the conditions of PCR cycles were 1 cycle at 95°C for 2 min. as initial denaturation, 30 cycles consist of denaturation at 95°C for 30 sec., annealing at 56.2°C for 30 sec. and extension at 72°C for 1 min. and then followed by 1 cycle of final extension at 72°C for 7 min. Then the PCR products seen by using UV-light Transilluminator after agarose gel electrophoresis.

Fagellin extraction and purification

Flagellin was purified from *P. aeruginosa* according to the procedure described by (17) with some modifications as follows: The activated bacteria was cultured on Brain Heart Infusion broth (1000 ml) and incubated in shaking incubator at 37°C for overnight until the absorbance at 600 nm reached to 0.6. Then centrifuge the bacterial cultures at 5000×g for 10 min. After that, washing the pellets by re-suspended it in 200 ml PBS / PH=7.4 and repeat this steps once again, and then resuspended in 50 ml from the same buffer. To monomerize the filaments and separate the flagellin, the suspension was stirrer 1000 rpm on magnetic stirrer for 45 min. at 4°C. After that separate the flagellin by centrifuge at 4500xg speed for 30 minutes to take the supernatant that contain the flagellins. Then precipitated the protein from the aqueous phase by slowly addition of 70% ammonium sulfate until the solution reach to the saturation for 1 hrs. at 4°C.

Protein Ultrafiltration

Proteins concentrating, infiltrating, and buffer exchanging by using ultrafiltration centrifugal devices called Protein concentrator 10 KD & 30 KD; Pierce™ Protein Concentrator 10 K & 30 K MWCO, 5–20 mL, #88527 & #88529 respectively (18). It contain polyethersulfone (PES) membrane, which allows for processing of volumes between 5 mL and 20 mL. It was done according to the instructions manual. Then, the protein absorption was measured using special spectrophotometer at 270 wave length.

Endotoxin Removal

All gram negative bacteria contain a structural components, biologically active, of the outer membrane called lipopolysaccharides (LPS), this LPS consider the endotoxin of these bacteria (19). The presence of the endotoxin in a recombinant protein or other type of purified protein in a small amounts when use as a vaccine or other uses can cause many side effects in the host organism as shock and

tissue injury may be lead to death of the organism; So that is important to minimize the endotoxin from the injectable substance to an acceptable amount for preclinical use (20). In this study this process was done by using special column "(Pierce High Capacity Endotoxin Removal Spin Column, 1 ml, #88276)" according to the manufacturer instructions (21). This endotoxin removal column contains ε-poly-L-lysine (porous cellulose beads) its surface modified to become with high affinity to endotoxins to eliminating it. Then measure the protein absorption again using special spectrophotometer at wave length 270 nm.

Endotoxin detection

The amount of the remaining endotoxin was detected by LAL Chromogenic assay using Endotoxin detection kit (Pierce™ Chromogenic Endotoxin Quant Kit, #A39552S; according to the manufacturer instructions (22,23). This Kit is an efficient, quantitative endpoint assay that uses amebocyte lysates derived from blood of the horseshoe crab to quantitate endotoxin in protein, peptides, antibodies or nucleic acid samples. Amebocyte lysates are widely used as a simple and sensitive assay to detect the endotoxin (lipopolysaccharide) in a gram negative bacteria.

When the amebocyte lysate expose to endotoxin that lead to activation many factor as Factor C, B and percolating enzyme that a catalyze the colorless chromogenic substrate to release a yellow substance called p-nitroaniline (pNA) that can measured photometrically at 405 nm. The intensity of color is proportional to the amount of endotoxin in the tested sample, that calculated by a standard curve.

Detection the presence of flagellin By SDS-PAGE

After flagellin purification and endotoxin removal, the presence of protein and its purity level was confirmed by electrophoresed in poly acrylamide gel using SDS-PAGE technique in %10 separating gel.

Results and Discussion

Flagellin gene (*fliC*) Detection in *P. aeruginosa* by PCR:

In this study, specific primers were used for *fliC* gene previously mentioned, for identification the type of flagellin in *P. aeruginosa* isolates by using a PCR technique was give variable product size for two types; type a at 1020bp, and type b at 1250 pb, after the optimum condition for this primers in PCR reaction.

After using the specific primers for *fliC* gene, for identification the type of flagellin in *P. aeruginosa* isolates using conventional PCR, The results of PCR Show the two types of flaellin in different strain; type A at 1020 bp, and type b at 1250 pb, as shown in (Figure 1).

Detection of flagellin protein and evaluate endotoxin contaminant by UV-spectrum:

After flagellin purification by using 70% ammonium sulfate and special ultrafiltration tube, the UV-spectrum of the sample was measured before and

after endotoxin removal to show the presence of the protein at 280 nm (24). The absorbance of other contaminants detected with another wavelength showed that acceptable protein level and the endotoxin (LPS) was decreased (at 190-250 nm) using the endotoxin removal kit as shown in (Figure 2).

Endotoxin detection

After removal the endotoxin from the purified protein by using "Pierce High Capacity Endotoxin Removal Spin Column, 1 ml" and measuring the concentration of endotoxin by a LAL Chromogenic assay according to the standard curve, The results shows that there are decreasing of endotoxin to the acceptable levels to preclinical use (less than 2.2 EU/ml) as shown in Figure 3 (20).

Detection the presence of flagellin protein and determine its purity by SDS-PAGE:

The result of SDS-PAGE technique show the two types flagellin proteins; type a flagellin 39 KDa and type b flagellin 45 KDa (25) in a different strain with high purity as shown in (Figure 4).

Conclusion

This research focused on isolation and purification of flagellin protein from *P. aeruginosa.* The study showed that the method used to isolate flagellin protein was highly acceptable that considered cost effective, highly acceptable, considered cost-effective, few steps required, few equipment with acceptable purity, and decreased endotoxin (LPS) for preclinical use.

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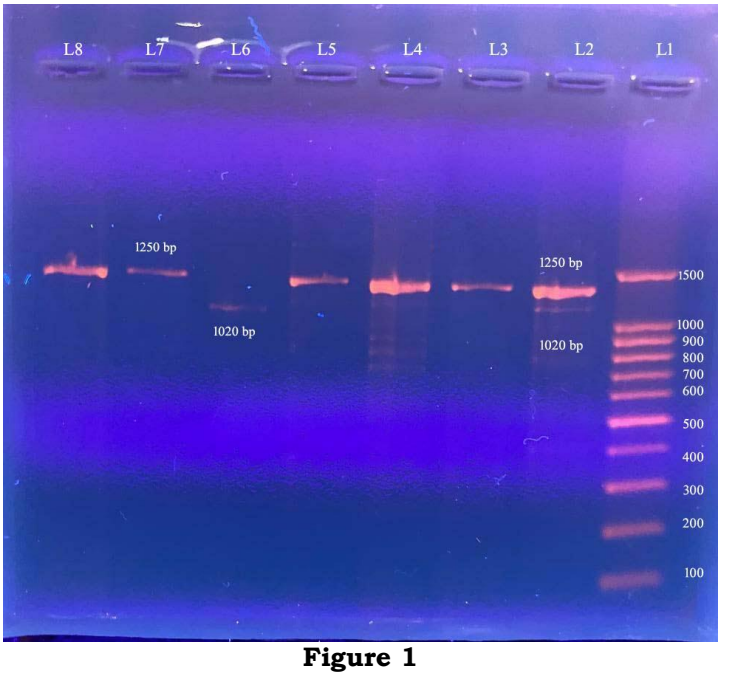

Figure 1

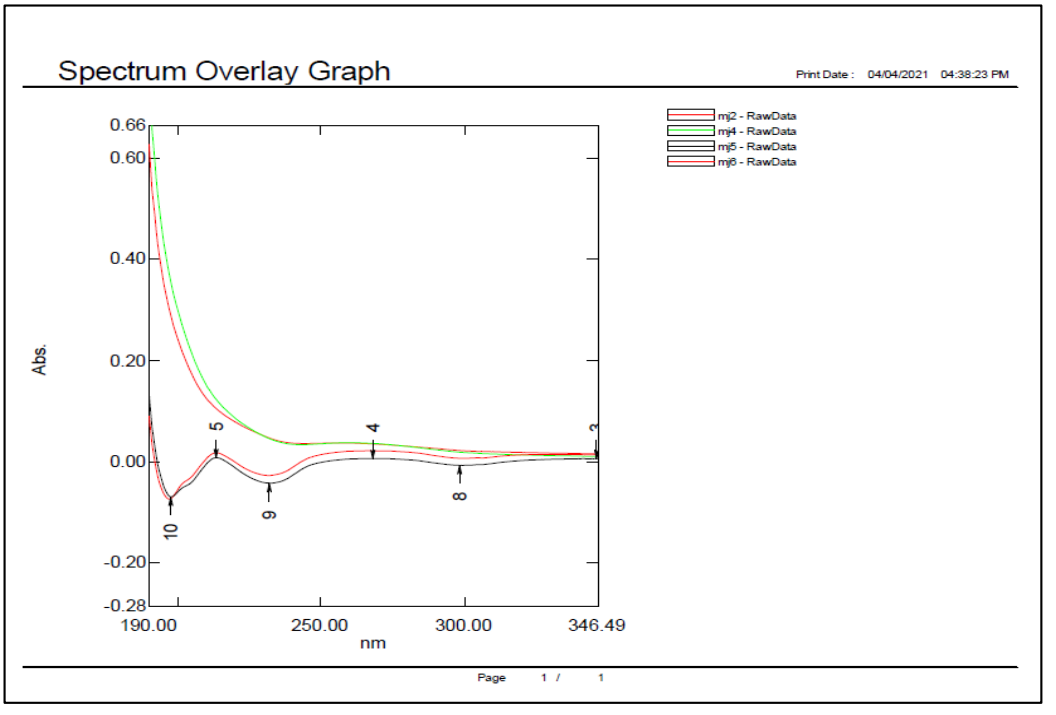

Figure 2

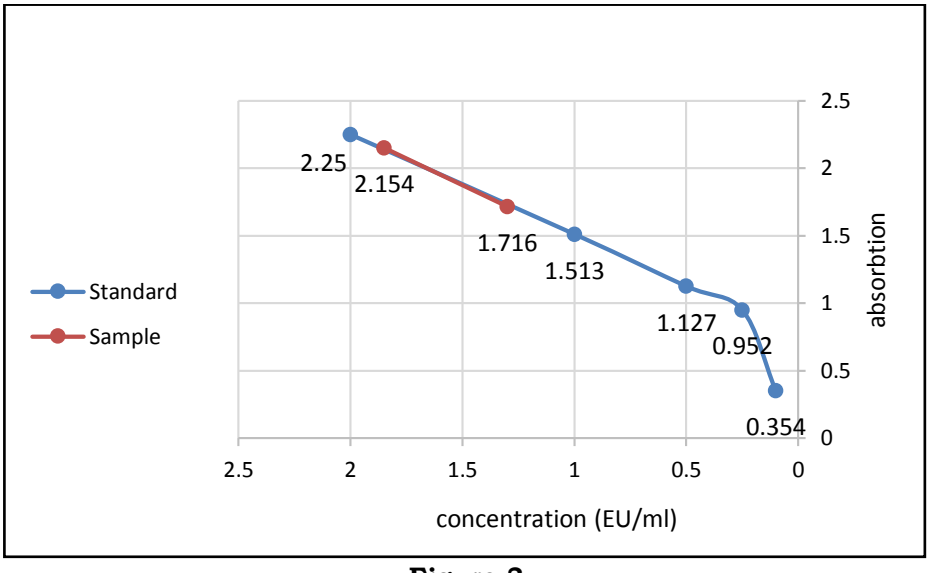

Figure 3

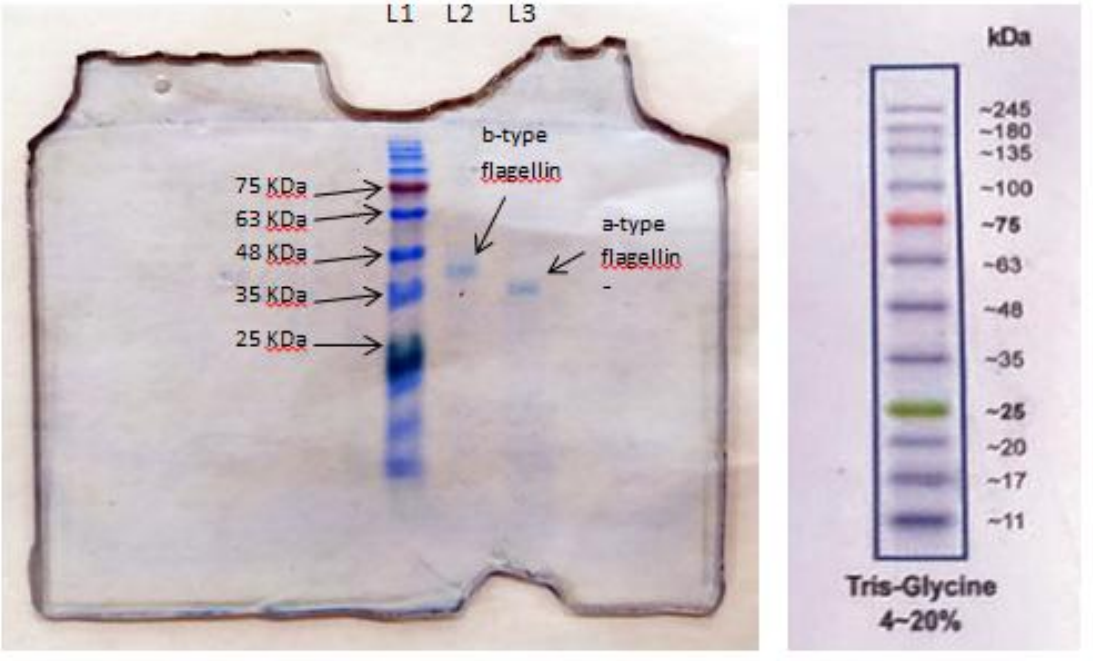

Figure 4

Figures Legends:

Figure 1: The gel of agarose (1.5% conc.) under UV light after electrophoresis of amplified fliC gene PCR products for 60 min. at 70 V. using ethidium bromide stain. L1: DNA ladder 100 bp. L2:Two band with size 1020 & 1250 pb that represent fliC gene type a and type b respectively. L3-5,7,8: band with size 1250 pb that represent type b fliC gene. L6: band with size 1020 pb that represent type a fliC gene.

Figure 2: UV-spectrum of purified flagellin from *P. aeruginosa*. Lines mj2 and mj4 show the spectrum of two purified protein samples before endotoxin removal. Lines mj5 and mj6 show the spectrum of two purified protein samples after endotoxin removal.

Figure 3: Standard curves for Pierce endotoxin quantitation kits

Figure 4:. Polyacrylamide gel after electrophoresis purified protein from *P. aeruginosa* by SDS-PAGE technique. L1: Protein ladder, L2: type b flagellins 45 kDa., L3: type a flagellins 39 kDa.