Abstract

Background: Type 4 pili (T4P) is an important virulence factor of *Pseudomonas aeruginosa* (*P. aeruginosa*). T4P pass the outer membrane through a large oligomeric channel made of a single PilQ protein that is most highly conserved at their C-termini. To develop a functional vaccine that can be used in clinical application, the secretin domain of the PilQ (PilQ<sub>380-706</sub>) was produced as a recombinant protein.

Methods: A 981 bp fragment of C-terminal of the pilQ secretin (pilQ<sub>1138-2118</sub>) from was designed into the prokaryotic expression vector pET28a. The presence of the pilQ<sub>1138-2118</sub> gene in the recombinant construct (pET28a/pilQ) was assessed by double digestion and PCR. After transformation, expression of the recombinant PilQ was induced by addition of IPTG. The expressed recombinant protein was purified by a modified method using a HisTrap affinity column and finally confirmed by SDS-PAGE. The functional activities of the produced PilQ<sub>380-706</sub> confirmed by Western blot analysis and twitching inhibition assay.

Results: The PCR and enzymatic digestion results showed the presence of the pilQ<sub>1138-2118</sub> gene in the construct. The protein electrophoresis showed that the molecular weight of the recombinant PilQ<sub>380-706</sub> is approximately 37 kDa. The Western blot analysis confirmed the specificity of specific IgG against the PilQ<sub>380-706</sub> protein. The PilQ<sub>380-706</sub> protein showed high biological activity in all of these standard assays.

Conclusion: Since, the PilQ<sub>380-706</sub> protein plays an important role in the biogenesis of pili; and thus, the primary establishment of *P. aeruginosa*; it seems that it can be used as a candidate vaccine or an adjuvant in the future studies.

Keywords: *Pseudomonas aeruginosa*, Proteins, Secretin

Introduction

*Pseudomonas aeruginosa* (*P. aeruginosa*) as an opportunistic human pathogen has a remarkable capacity to cause disease in susceptible hosts. It is the major colonizing microbial pathogen for Cystic Fibrosis (CF) patients and a common infectious agent in nosocomial infections, such as patients with a severe burn, cancer, transplantation, AIDS, and other immunocompromising conditions. Despite improvements in antibiotic therapy, *P. aeruginosa* shows inherent and acquired resistance to many antimicrobial agents. The pathogenesis of *P. aeruginosa* infections is multifactorial and is being affected by a complex of virulence factors; hence, it has made vaccine development difficult. Bacterial attachment is an initial and a critical step for the establishment of infection that involves bacterial adhesins and host receptors. One of the most important adhesins in *P. aeruginosa* is pili.

Type IV pili (T4P) are the most common type of bacterial pili and are thin, long, flexible, and retractable protein filaments. T4P are polarly localized, filamentous surface appendages present at the cell surface of a broad range of pathogenic and environmental bacterial species. This adhesive cell surface structure is the prominent virulence factor that essential for initiation
of infection by mediating attachment to host cells, where non-piliated strains were reported to exhibit a 90% decrease in their ability to bind human alveolar cells, and also mutant strains that are unable to produce T4P are attenuated in virulence. Furthermore, another study revealed that piliated strains caused 28%-96% more cases of P. aeruginosa pneumonia as compared to non-piliated strains in a mouse model of infection. T4P play an important role in many processes including bacterial locomotion known as twitching motility, aggregation, infection by pilus-specific bacteriophage, DNA uptake, attachment to biotic and abiotic surfaces, host cell invasion and biofilm maturation.

The pilus fiber is composed of hundreds of copies of PilA (or pilin, the major structural subunit) that are encoded by an operon that positively control by the algR regulator. The pilin monomer can be divided into three domains: a highly conserved hydrophobic N-terminal α-helix region; a hypervariable central region; and a semi-conserved C-terminal region containing β-strands. The C-terminal Receptor Binding Domain (RBD) of P. aeruginosa pilin is a suitable candidate for a peptide vaccine. The RBD contains a disulphide-bonded loop (DSL) that structurally is highly conserved among T4P of all species of P. aeruginosa, although the size of the DSL (from 12-31 amino acids) and its sequence is varied among pilin alleles.

The monoclonal antibody studies revealed that the C-terminal DSL of the pilin subunit mediates attachment to epithelium receptors, this finding suggests that PilA itself acts as both a major structural subunit and an adhesion. Finally, Type IV pili has a common receptor among all strains of P. aeruginosa; however, the sequence diversity presents a considerable obstacle to the development of a protective RBD-based vaccine targeting the T4P.

Pili are rapidly extended and retracted via a most powerful molecular machine that organized with four subcomplexes: the cytoplasmic motor subcomplex (consisting of PilBTUCD), the inner membrane alignment subcomplex (PilMNPQ), the outer membrane secretin pore subcomplex (PilQ and PilF), and the pilus itself (or PilA). There are significant structural and functional similarities between this pilus assembly apparatus and type II secretion system. T4P passes the outer membrane through a large oligomeric channel and makes a single protein. The PilQ (77 kDa; ORF PA5040) that encoded by the highly conserved pilMNPQ operon is a member of the so-called “secretin” family required for configuration of the outer membrane pore through which the pilus is extruded.

The PilQ protein that is essential for T4P biogenesis consists of five conserved domains; Secretin N (380-449), Secretin (549-705), STN (306-354), HoiQ (1-707) and AMIN (63-123). The secretin domains (Secretin N and Secretin) of the PilQ are more highly conserved at their C-termini. This region facilitates the passage of folded proteins, filamentous phage particles, DNA, and other macromolecules across the outer membrane. The secretin domain of PilQ (that’s mean PilQ(380-706)) was chosen as a new antigen and designed into expression vector pET28a.

In the present study, we designed a chimeric plasmid contains the pilQ1138-2118 gene, which codes the immunologic domains of PilQ secretin (the C-terminal domain of the PilQ). To the best of our knowledge, for the first time, we report the purification and characterization of a novel recombinant PilQ (r-PilQ(380-706)) from P. aeruginosa. Furthermore, our data suggest that the protein has biological activities in both in vivo and in vitro conditions.

Materials and Methods

Bacterial strains, plasmids, and media

Escherichia coli (E. coli) strains Top10F and BL21 (DE3) were used as preservation and expression hosts. The P. aeruginosa laboratory strain PAO1 (that kindly provided by Dr. Abdi from Department of Microbiology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran) were performed. The recombinant plasmids pET28a/pilQ1138-2118 Synthesized by Biomatik Corporation (Cambridge, Ont., Canada). All enzymes for DNA manipulations were obtained from NEB (USA), The Ni²⁺-NTA agarose was purchased from Qiagen (USA). The HRP-conjugated goat anti-rabbit IgG and Protein A/G agarose were obtained from ThermoFisher (formerly Invitrogen, USA). The strains were cultured in LB broth or on agar (HIMEDIA, India) at 37°C with or without 30 µg kanamycin/ml (Bioscience, Canada).

Construction of the expression vector

The pilQ1138-2118 gene was inserted into the E. coli expression vector pET28a, in frame with a T7 promoter, kanamycin-resistant gene and the C and N-terminal six-His-tagged sequences. The gene containing BamHI and HindIII sites at the 5’ and 3’ ends, respectively. In the designation of the construct, we have inserted a start codon ATG immediately after the BamHI site (ggatccATG) of the pET28a vector, resulting in the correct framing of the gene of the insert. After transformation of the recombinant vector (pET28a/pilQ1138-2118) into E. coli Top10F competent cells, transformants were screened on LB plates supplemented with 30 µg kanamycin/ml. The recombinant vector was extracted from E. coli Top10F using plasmid extraction kit (Bioneer, Korea) according to manufacture instruction.

Confirmation of the recombinant vector

The pET28a/pilQ1138-2118 vector was verified by polymerase chain reaction (PCR) and restriction enzyme digestion. The vector was treated with the restriction endonucleases BamHII and HindIII (Jena Bioscience Kit, Germany) according to manufacture instruction. The specific primers were designed for the pilQ1138-2118 sequence of the P. aeruginosa PAO1 strain from NCBI (Gene ID: 880962). The gene was amplified from pET28a/pilQ1138-2118 vector using the following specific primers.
primers: Forward: 5’-CCA GGT GAA CTA CGC CAA GG- 3’ and Reverse: 5’-CGG TGT CAG GAA AAC CAG AAG T-3’, which incorporated by BamHI and HindIII restriction sites, respectively. Amplifications were performed in 25 µl volumes containing 50 pM of each primers, 1X PCR master mix, and 25 ng of the vector. The PCR was performed in a master gradient thermocycler (Bio-Rad, USA). Template DNA was denatured at 94°C for 4 min prior to the start of PCR cycling, which consisted of denaturation at 94°C for 46 s, primer annealing at 61°C for 1 min, extension at 72°C for 1 min, repeating for 30 cycles followed by a final extension at 72°C for 10 min. The digested fragments and PCR product were separated by 1.2% (w/v) agarose gel electrophoresis.

Expression and isolation of inclusion bodies

In order to overexpress the protein, the recombinant construct pET28a/pilQ1138-2118 was transformed into BL21 (DE3) and plated on LB agar containing kanamycin (30 µg/ml). To optimize the induction conditions, the colonies carrying pET28a/pilQ1138-2118 were grown in 5 ml of LB medium supplemented with kanamycin at 22°C. At OD600 nm of 0.8, expression of the r-PilQ380-706 was induced by addition of IPTG (BIO-SYNTH, Switzerland) to a final concentration of 1 mM. After 0, 1, 2, 3, 4 and 5 hr of induction, cells were harvested and the induced level of r-PilQ380-706 was determined by 12% SDS-PAGE electrophoresis.

For isolation of inclusion bodies, an overnight culture of E. coli BL21 (DE3) cells harboring pET28a/pilQ1138-2118 was diluted 100-fold in LB medium (1 liter) containing kanamycin and incubated at 22°C with shaking. When the OD600 of the culture reached 0.8, the promoter of the recombinant vector was induced by the addition of IPTG to the final concentration of 1 mM. After 4 hr, the induced cells were harvested by centrifugation at 8500×g for 10 min at 4°C and suspended in lysis buffer [20 mM sodium phosphate, 500 mM NaCl, 6 M guanidine hydrochloride, pH=7.4]. The contaminant proteins were washed using native wash buffer [20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH=8.0]. Finally, bound proteins were eluted in native elution buffer [20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH=8.0]. The purified r-PilQ380-706 was dialyzed against phosphate buffered saline (PBS, pH=7.4) for imidazole removal and analyzed by 12% (w/v) SDS-PAGE followed by Coomassie brilliant blue G-250 staining. The protein concentration was quantitatively measured by using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) and Bradford protein assay using standard albumin (Sigma, USA).

Preparation and purification of anti r-PilQ380-706 IgG

To determine the immunogenic nature of purified r-PilQ380-706, the female New Zealand white rabbits (Razi Vaccine and Serum Research Institute, Karaj, Iran) were immunized with 400 µg of the r-PilQ380-706 protein administered subcutaneously and boosted twice with 200 µg with 2 weeks intervals. The rabbits were anesthetized intramuscularly with an injection of a mixture of xylazine (10 mg/kg) and ketamine (50 mg/kg). The rabbits were bled prior to immunization and 2 weeks after the last immunization. Sera were collected from the retracted clot, clarified by centrifugation (2500×g) and then aliquoted and stored at -20°C. The rich fractions pooled and the specific IgGs (except IgG3) purified by using protein A/G agarose (Invitrogen, USA) according to the manufacturer’s instructions with modifications. Purifications were performed under denaturing and renaturing conditions (hybrid conditions). Briefly, after applying the sample to the column and washing with denaturing binding buffer [20 mM sodium phosphate, 500 mM NaCl, 8 M urea, pH=7.8], a linear gradient of urea from 7 M to 0 M of refolding buffer [20 mM sodium phosphate, 500 mM NaCl, 5% (v/v) glycerol, pH=6.0] was used at flow rate of 0.6 ml/min. The contaminant proteins were washed using native wash buffer [20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH=8.0]. The purified r-PilQ380-706 was dialyzed against phosphate buffered saline (PBS, pH=7.4) for imidazole removal and analyzed by 12% (w/v) SDS-PAGE followed by Coomassie brilliant blue G-250 staining. The protein concentration was quantitatively measured by using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) and Bradford protein assay using standard albumin (Sigma, USA).

SDS-PAGE electrophoresis and Immunoblot analysis

The bacterial pellets and purified protein were separated by SDS-PAGE. The samples were directly resuspended at a 2:1 ratio with 3× SDS-PAGE sample buffer. in an appropriate volume of sample buffer. The discontinuous gel consisted of a 3% stacking gel and a 12% resolving gel which was run on a vertical electrophoresis unit (Mini PROTEAN 3 cell, Bio-Rad). To determine the functional activity, the purified r-PilQ380-706 protein was electrophoresed, and then transferred onto a PVDF membrane (Hi-bond Amersham Biosciences, USA) by a Bio-Rad apparatus at 25 V for over-
night. The membrane was blocked with 5% non-fat skim milk in TBST buffer (Tris buffer saline contain 0.1% Tween-20) for 2 hr at RT. After incubated with rabbit anti r-PilQ380-706 IgG (1:5000 diluted in blocking buffer) for 1 hr at RT, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG for 1 hr at RT. The membrane was then washed 5 times with TBST for 5 min each. Finally, it was developed by adding 3, 3′-diaminobenzidine (DAB) solution (Sigma, USA) allowing it to incubate until bands were seen. The reaction was stopped by rising the membrane with water.

Twitching inhibition assay

To verify the functionality of the r-PilQ380-706 specific polyclonal IgG, the twitching inhibition assay was carried out by Castric et al \(^{17}\) as follows. Different concentrations (0.1, 0.2 and 0.3 µg) of specific rabbit anti r-PilQ380-706 IgG (filter-sterilized) were added to LB broth (containing 1% (v/v) agar), which was poured into a 15×90 mm plastic Petri dish. After solidification, the plate was dried for 6 hr at room temperature. A single colony of the P. aeruginosa PA01 strain to be tested was stab-inoculated with a toothpick to the bottom of the plates. After omitting an 18 hr incubation at 37°C, the diameter zone of growth of different strains obtained at the interstitial surface of the agar and the plate was measured. For each assay, triplicate plates were examined.

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) and Student’s t-test (StatView). Statistical analysis was performed using the software GraphPad Prism version 6.0 for Windows, (GraphPad Software, San Diego, CA, USA). All data of this study are expressed as mean±SD. The p-values less than 0.05 was considered to be statistically significant.

Results

Confirmation of the pET28a/pilQ1138-2118 construct

The coding sequence of the secretin domain of pilQ (pilQ1138-2118) was constructed in the pET28a expression vector. Transformants were characterized by enzymatic digestion. The recombinant plasmid, pET28a/pilQ1138-2118, was extracted and its orientation confirmed by digestion with two restriction enzymes that mentioned above. The target fragments with the expected sizes are shown in figure 1. DNA gel electrophoresis of the PCR product resulted in single 981 bp band, which confirmed amplification of pilQ1138-2118 gene (Figure 1). Sequence analysis of recombinant pET26b/pilQ1138-2118 confirmed that there are no amplification errors and that construction was accurate.

Expression and purification of r-PilQ380-706

To construct an expression system, the coding sequence of PilQ380-706 whose theoretical molecular size is approximately 35 kDa, was constructed into expression vector pET28a to express a recombinant protein.

The recombinant plasmid, pET28a/pilQ1138-2118, was transformed into E. coli BL21 (DE3). The His-Tagged recombinant protein was purified from inclusion bodies by using Ni\(^{2+}\)-affinity chromatography. Results from SDS-PAGE analysis of expression products showed that the PilQ380-706 protein expressed 4 hr after induction with IPTG. The expression product of the protein was approximately 37 kDa in molecular size (Figure 2). The purified protein was confirmed by western blotting as showed in figure 3. The yield of the purified PilQ380-706 protein was about 2.18 mg per liter of cul-
ture media. In term of measured protein, no significant
difference was observed between NanoDrop and Brad-
ford protein assay.

Production of PilQ380-706-specific IgG and specificity analy-

sis

Polyclonal antibodies against r-PilQ380-706 were pro-
duced in rabbit and finally the specific IgG was puri-
fied by using protein A/G agarose (Invitrogen, USA)
according to manufacturer's instruction. As shown in

figure 4, β-Mercaptoethanol as a reducing agent break

the hinge-region disulfide bonds and thus antibodies
will dissociate into the heavy (51.4 kDa) and light
(24.9 kDa) chains, respectively. To determine the spec-
ificity of the antiserum raised against purified r-PilQ380-
706, western blot analysis was performed. The total cell
extracts (induced and non-induced) and the purified
PilQ380-706 protein were immunoblotted and then hy-
bridized with specific polyclonal IgG. Addition of
HRP-conjugated goat anti-rabbit IgG showed that the
PilQ380-706 protein was substantially expressed by using
the pET28a/pilQ1138-2118 expression vector when IPTG
was added at the early-exponential phase of growth and
collecting the cells 4 hr after induction (Figure 3).

Overall, our results indicat ed that the rabbit produced
antibodies are highly specific to detect the r-PilQ380-706.

Twitching inhibition assay

Immunized and non-immunized rabbit sera were
evaluated in the twitching inhibition assay for their
biofunctional activity to inhibit the motility of PAO1
strain of P. aeruginosa. In this assay, NRS was used as
control group. As shown in figure 5 and table 1, the r-
PilQ380-706 IgG was able to inhibit the motility of
P. aeruginosa PAO1 (b to d, respectively) compared with NRS (a).

Discussion

Among the various recombinant immunodominant
antigens identified as a candidate vaccine against P.
aeruginosa, the outer membrane proteins have shown
promising potential. Hence, we tried to improve the
purification conditions to obtain the recombinant pro-
tein in the pET28a vector system. In the present study,
the secretin domain of the PilQ gene (pilQ) from \textit{P. aeruginosa} was designed into pET28a vector. This vector is one series of vectors without signaling regions allows accumulation of the PilQ protein (insoluble form). These types of proteins have been efficiently de- and renatured. In addition, two 6×His-tag was designed at the N- and C-terminus of the r-PilQ protein encoding sequence. His-tag, a small purification partner, has been designed into pET28a vector to decrease the time and cost of protein purification procedures without affecting protein well folding and bioactivity. The pET system was chosen because it is a very powerful system developed especially for the cloning, expression and purification of recombinant proteins in \textit{E. coli} and also has been utilized to overexpress exogenous proteins for decades. The PilQ protein was expressed in BL21(DE3), carrying an inducible chromosomal gene for T7 RNA polymerase, which is controlled by an IPTG-inducible lacUV5 promoter. pET-series vectors also contain a lacI gene that provides lac repressor molecules to downregulate both the lacUV5-controlled chromosomal T7 RNA polymerase and the T7lac promoter. In the previous study, Fakhri et al cloned and expressed the conserved C-terminal fragment of the PilQ protein (PilQ) from \textit{Neisseria meningitidis} into pET28a vector. They demonstrated that, when the recombinant vector transform into prokaryotic expression system, high level of protein is produced following nickel affinity chromatography.

Most of the recombinant proteins thought aggregate as inclusion body, but can be solubilized and purified using hybrid condition. The significant increase in the yield of protein extraction from the inclusion bodies can be achieved by the addition of guanidine hydrochloride (G-HCl) as a solubilization agent. In this condition, the solubilized recombinant protein bind and wash under denature condition, and wash and elute under native conditions. Use of 6 M G-HCl alone as strong denaturant was sufficient for the solubilization of protein from inclusion bodies. In this study, we made the use of optimum concentration of non-ionic detergent (Triton X-100) that help to efficiently purify the r-PilQ protein from inclusion bodies. The use of 1% Triton X-100 helps to improve the lysis conditions of cells for further solubilization of inclusion bodies. Furthermore, after three rounds of washes under native conditions, the proportions of unrelated proteins were considerably decreased (data not shown). In addition, 5% (v/v) glycerol was added in the refolding buffer as it enhanced the stability and efficiently elevating the yield of protein.

In recent years, high-throughput protein-refolding techniques have been developed for renaturation of inclusion bodies. These include three methods such as dilution, dialysis or solid-phase separation for renaturation of inclusion bodies. In the present study, for improvement of the refolding process, we have selected dilution and dialysis methods. In on-column purification, we used a decreased gradient of urea for the gradual removal of urea and renaturation of recombinant protein. This washing process followed by dialysis (with buffer exchange), in which there was no protein precipitation and aggregation. Our efforts at refolding of the solubilized proteins using dilution and dialysis methods were led to effectively refolded desired recombinant protein. We found that after chromatography by Ni-NTA agarose, unrelated proteins further decreased, this was lead to an increase in the refolding yield and purity. In the present study, we not only evaluated the efficiency of pET28a vector for the expression of the r-PilQ and also simultaneously developed a highly reproducible and efficient procedure for purification and scalable production of the recombinant protein with high purity. The procedure developed here may be useful in the efficient purification of other recombinant proteins highly expressed in \textit{E. coli} as inclusion bodies. Generally, the protein (PilQ) tends to be expressed as inclusion bodies at lower temperature, but the rate of expression is more slowly for correct folding. Now that culture under lower temperatures are beneficial to stabilization of structure and expression of soluble protein, and the increase of temperature did not significantly enhance PilQ expression (data not shown), the following induction was carried out at 22 °C. Overall, after several attempts to determine the optimal conditions, the highest amount of PilQ protein was produced by induction with 1 mM IPTG at 22 °C for 4 hr.

The immunoreactivity of purified r-PilQ under modified conditions was examined in vitro by twitching inhibition assay. The twitching inhibition assay results show that the antiserum raised against the r-PilQ can inhibit cell motility of \textit{P. aeruginosa} PAO1 in vitro. Immunoblot analysis demonstrated that the r-PilQ-specific polyclonal IgG could detect the recombinant protein expressed in a prokaryotic cell (\textit{E. coli} BL21). These findings indicate that the r-PilQ preserved correct folding. Since motility has been exhibited to be an important virulence factor in microbial pathogenesis, therefore, disruption of such a function by neutralizing and immobilizing antibodies may prove to be an advantageous prophylactic measure against pathogenic bacteria. These tests confirmed the bioactivity of the purified recombinant protein. Thus, the use of these reagents in the modified protocol does not have any adverse affects on the bioactivity of the protein. In the recent study, showed that the absence of twitching motility of \textit{P. aeruginosa} is correlated with the lack of PilQ multimer. We believe that, this is the first report on the expression and purification of the secretin domain of the PilQ protein with a His-tag in bacterial expression system. It is suggested that the r-PilQ could contribute as a vaccine or an adjuvant to control \textit{P. aeruginosa} infection.
Conclusion

In conclusion, the present study described a modified method for expression, purification and refolding of r-PilQ380-706 from P. aeruginosa in E. coli. The recombinant protein was expressed in the form of inclusion bodies under the pET28a expression vector. Here, we developed a reproducible and simplified method to achieve significant yields of the protein. The purification of r-PilQ380-706 was done under the modified hybrid condition. The procedure developed in this study may be useful in the efficient purification of other recombinant proteins expressed in E. coli as inclusion bodies. This recombinant protein was biologically active and recommended to be used as a vaccine or an adjuvant.

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