Original Article

Design and Production of Arginine Deiminase-Azurin Recombinant Fusion Protein from Pseudomonas *aeruginosa* and its Confirmation by Western Blot

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Abstract

Background: *Pseudomonas aeruginosa* is a common Gram-negative, rod-shaped bacterium that has a unique genome that allowed the bacteria to produce various enzymes and proteins. Azurin and arginine deiminase are low molecular weight proteins that produced by P. aeruginosa. These proteins can be used alone or in combination together in order to become effective in cancer therapy or inhibiting of metastasis. This study aimed to design, express and purify the Azurin and Arginine Deiminease recombinant fusion protein.

Materials and Methods: The sequences of Azurin and arginine deiminase from *P. aeruginosa* were studied for synthesis in a pET28a vector. The recombinant plasmid was transfected into the E.*coli* BL-21 strain and expression was induced by isopropyl-β-D-thio galactopyranoside (IPTG). The fusion protein expression was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Purification of the recombinant product was performed by the Ni-NTA chromatography column, obtained product analysis with SDS-PAGE and Western blot technique.

Results: Cloning was confirmed by observing bands from the enzyme digestion. The protein band with a molecular weight of 65 kDa on the SDS-PAGE gel was an indication of the correct expression of the protein. The single-band of this recombinant protein was confirmed by the western blot technique.

Conclusion: In this study, due to the successful production of arginine deiminase-azurin fusion protein, and considering the separate anti-cancer properties of these compounds, which have been reported in previous studies, it is suggested that immunological assessments and effects of this fusion protein in different cancerous cell line be investigated.

Keywords: *Pseudomonas aeruginosa*, Azurin, Arginine deiminase, Fusion protein

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Introduction

Pseudomonas aeruginosa is a gram-negative, rod-shaped bacterium which is one of the main causes of nosocomial infections, especially in cystic fibrosis patients, burns, and people with immune deficiency[1,2]. The variation of *P. aeruginosa* pathogenicity indicates that this bacterium has a unique genome and has a tremendous enzymatic power, which allows the bacteria to survive in different conditions[3]. 10% of the genome of this bacterium is organized in pathogenicity islands, which

provides the most pathogenic variation for *P. aeruginosa* [4]. *P. aeruginosa* expresses a variety of cellular products, such as adhesins, proteases, phospholipase, exotoxins, which are related to its pathogenesis[5]. Azurin and Arginine deiminase as two proteins produced by *P. aeruginosa* play a major role in pathogenesis of this bacterium[6,7].

Azurin is a low molecular weight protein (128 amino acids -14 kDa) and a member of the cupredoxin family produced by *P. aeruginosa* [7]. This protein has an effect on the electron transfer cycle during the respiration of germs and carries an electron in the respiration process[8]. Azurin has anti-cancer and antiviral properties[9].

Arginine deiminase widely used as a therapeutic agent for the treatment of arginine-auxotrophic tumors, such as hepatocellular carcinomas and melanomas[10-13]. Arginine deiminase extracted from *P. aeruginosa* is called Pa-ADI[14]. Active Arginine deiminase as a tetramer protein of about 46 kilodaltons per monomer and catalyzes irreversible hydrolysis of L-arginine to Citrulline and Ammonium[15,16].

Pseudomonas proteins and genes are used to design and produce fusion protein for pharmaceutical and therapeutic purposes[17-20]. The fusion protein made in the laboratory by combining different genes or parts of the genes from the same or different organisms. The produced fusion protein may yield different functions derived from each of its components[21]. Construction of recombinant fusion proteins is accepted as a means to increase the expression of soluble proteins and facilitating protein purification[22].

Considering the ability of this bacterium, like its different protein production, which is used in vaccination strategies, its pathogenesis antibiotic resistance which have been considered during treatment; in this study, we designed and produced azurin and arginine deiminase fusion protein from *P. aeruginosa* for the first time and provided an opportunity for further research.

Methods

Bacterial strains and materials: *E. coli* DH5α and *E. coli* BL21 (DE3) were purchased from Pasteur Institute of Iran, Tehran, Iran for cloning and expression experiments, respectively. Ni-NTA purification resin purchased from Qiagen™, South Korea. The *NcoI* and *HindIII* restriction enzymes were obtained from TAKARA, Japan. Kanamycin and Anti-poly-histidine antibody-HRP was purchased from Sigma-Aldrich, Germany.

Bioinformatics studies: To construct the fusion protein, *azu* and *arcA* gene sequences were obtained from NCBI website and the (GlyGlyGlySer) x2 sequence were used as a linker between two proteins; after codon optimization spatial shape, half-life and instability index of the fusion protein were determined using Raptor X Binding and Prot Param online software. The gene fragment was ordered to Biomatik Company (Canada) for synthesis in the pET-28a vector between *NcoI* and *HindIII* restriction enzyme sites.

Transformation of the recombinant plasmid: The recombinant pET28a-*arcA*-*azu* plasmid was transformed into E.*coli* DH5α competent cells by thermal shock method. Amplified plasmids were extracted using the plasmid extraction kit (Bioneer, Korea). The presence of *arcA*-*azu* gene sequence in the plasmid was confirmed by enzymatic double digestion; Digestion of the produced plasmids by *NcoI / Hind III* was carried out at 37ºC for 1.5 h to release the gene fragment and was detected by gel electrophoresis.

Expression and purification of the fusion protein: Induction and expression of the recombinant *arcA*-*azu* gene were carried out in E.*coli* BL21 (DE3) competent cells. A single colony from BL21 harboring the construct was inoculated in 1 Liter of Luria-Bertani broth (LB) culture containing kanamycin at final concentration 100 µg/ml. The culture was used for expression and when the optical density (OD at 600 nm) reached 0.6 nm, induction performed with 0.5 mM of IPTG at 37°C for 3 hours.

Bacteria were precipitated by centrifugation at 12000 rpm for 20 min at 4ºC and resuspended in a lysis buffer (50 mM NaH2PO4, 0.3 M NaCl, 8 M urea, pH 8.0). After the cells were lysed, the suspension was sonicated (5 cycles of 60 seconds pulses and 1-minute intervals) on ice and debris was removed by centrifugation at 14000 rpm for 12 min.

Ni-NTA affinity chromatography was used for the purification of the fusion protein. The supernatant of

the cell lysate was passed through the nickel–NTA column and the column was washed with decreasing urea concentration (from 0 to 8 M) and then the column was eluted with increasing imidazole concentration (concentration gradient of imidazole 150-500 mM). The expressed and purified fusion protein were analyzed by 12.5% SDS–PAGE and stained with the Coomassie Brilliant Blue R-250, was examined.

Antigenicity of the fusion protein: Antigenicity of the fusion protein was evaluated using western blot analysis. Western blotting was performed on 12.5% SDS-PAGE. The protein bands in SDS-PAGE were transmitted by electrical force to the nitrocellulose membrane (0.45 μm) using standard procedure. The nitrocellulose membrane was blocked with Tris-buffered saline (TBS) – skim milk 1 % for 2 h. Membrane was washed in Tris-Buffered Saline Tween-20 (TBS-T) for 3 times. According to the design of the histidine sequence at the end of the fusion protein, the Anti-poly histidine HRP antibody (Sigma) was used at 1 to 1000 dilutions in TBS-T. Finally, the nitrocellulose membrane was placed in 0.5 mg/ml 3, 3'- diaminobenzidine (DAB) solution containing 0.1% hydrogen peroxide, for 30 minutes, and the appearance of the desired protein band was investigated.

Results

The prediction of the fusion protein showed that the linker could keep two proteins apart (Fig. 1). Biochemical analysis of fusion protein showed that the molecular weight of the fusion protein was 65038 Dalton and the isoelectric point was 5.84. The aliphatic index of the fusion protein was 86/26 and the instability index was 35/86. The instability index indicates the sustainability of recombinant protein in such a way proteins with an index of less than 40 are categorized in Stable proteins.

Transformation of the recombinant plasmid: To confirm the transformation, cutting with *NcoI* and *HindIII* restriction enzymes with one cut site on the vector were used. The recombinant gene was detected at 1745 bp in the lower region of the linearized plasmid on the agarose gel 1% (Fig. 2).

Expression and purification of the fusion protein: The best level of expression was determined in IPTG 0.5 mM, temperature 37°C and 3 h post-induction incubation time. To evaluate the protein expression, The IPTG induced samples, as well as non-induced samples as the negative control, were analyzed on SDS-PAGE. The fusion protein band of the recombinant arginine deiminase-azurin protein was obtained after staining with Coomassie Brilliant Blue 250-R (Fig. 3).

The molecular weight of the recombinant fusion protein was 65.05 kDa. The presence of the induced band in the range of 65 kDa of protein corresponded with the expected molecular weight.

The existence of the Poly-histidine sequence at the end of the protein led to the use of a nickel affinity chromatography column to purify the protein. The expressed protein was mainly accumulated as inclusion bodies which purified by Ni2+ affinity chromatography under modified denaturing condition; for this purpose, the column was washed by using an

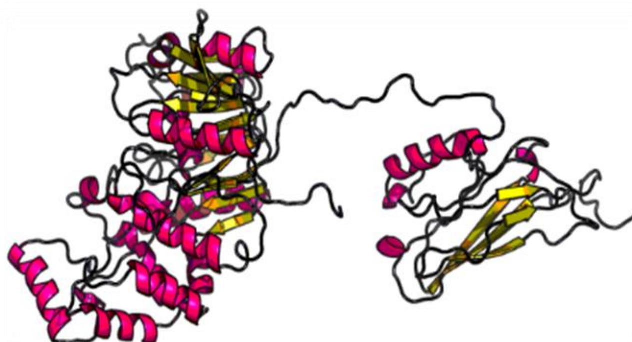

Figure 1. Prediction of the spatial structure of the fusion protein.

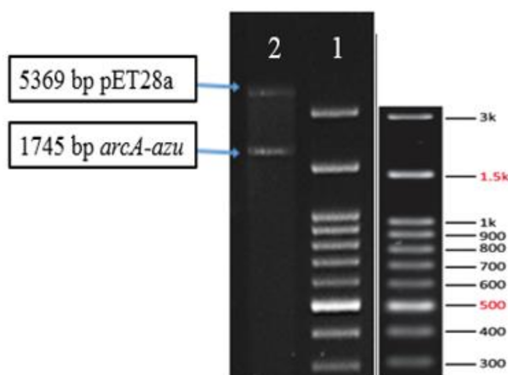

Figure 2. Confirmation of recombinant plasmid containing *arcA-azu* genes by double digestion; Lane 1: Molecular weight marker, Lane 2: Vector digested with *Nco*I and *Hind* III enzymes (recombinant gene with 1745 bp in the lower region of the plasmid).

elution gradient of 150 to 500 mM imidazole concentration and the purity of the protein was evaluated by SDS-PAGE (Fig. 4).

Antigenicity of the fusion protein: Western blot was used to evaluate the antigenicity of the produced fusion protein. The purified protein was electrophoresed in the SDS-PAGE gel (Fig.5 A), the bands transferred to nitrocellulose paper. A brown strip consistent with the location of the recombinant protein appeared on nitrocellulose membrane that confirmed the fusion protein reaction with antibodies (Fig.5 B).

Discussion

The fusion protein is a protein made from hybrid genes, which is created by joining two or more different genes. Studies showed that the obtained fusion protein may yield many distinct functions derived from each of its molecular components[21]. As a result, production of fusion protein production is very cost-effective compared to natural proteins. Also, the use of fusion protein in comparison to the use of each protein alone increases the chance of receiving and producing fusion protein simultaneously in the desired host cell.

In general, fusion protein has widespread use in biological research, such as protein purification[23], cancer treatments[24-26] and also use for enhancing the potency of cancer vaccine in the protein format[26,27], which has made this compound an important

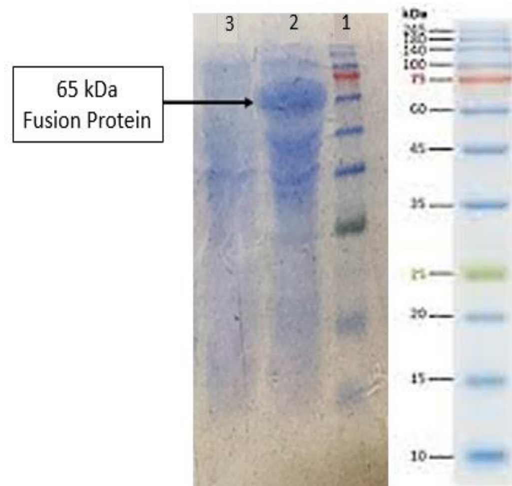

Figure 3. Evaluation of fusion protein expression in SDS-PAGE gel; Lane 1: Protein Molecular Weight Marker, Lane 2: Protein deposition of non-induced recombinant bacteria (Control Sample), Lane 3: Protein deposition of induced recombinant bacteria.

category of biologically active substances[28,29].

P. aeruginosa is a Gram-negative opportunistic pathogen that known to secrete the protein called azurin as a weapon against invaders as cancers, parasites and viruses. The production of such proteins by pathogenic bacteria could provide views into how a pathogen uses mechanisms and proteins as responds to impede other intruders for its own survival. The pharmaceutical capacity of these molecules like azurin in the industry as next-generation therapeutics was found recently. Azurin (14 kDa, 128 amino acids) is a copper-containing single-domain protein with a rigid β-barrel structure, produced by *P. aeruginosa*. Azurin rely on the stability of the p53 protein capable to destroy tumor cells and inhibits the development of the cancer. In nucleus of azurin enhances the intracellular levels of p53 and Bax, and triggers the release of mitochondrial cytochrome C into the cytosol. Caspase cascade activates by this process and initiates the apoptotic process[30]. Over the years, many studies have been done on the synthesis and production of azurin and peptides and the fusion proteins[31-33]. In a study, the azurin-cytochrome P450 fusion protein was designed using azurin from *P. aeruginosa* and cytochrome P450 from *Pseudomonas putida* in pET-21b vector and then transferred to E. *coli* BL21 and the fusion protein was obtained by chromatography column[34].

Arginine deiminase is another Pseudomonas protein

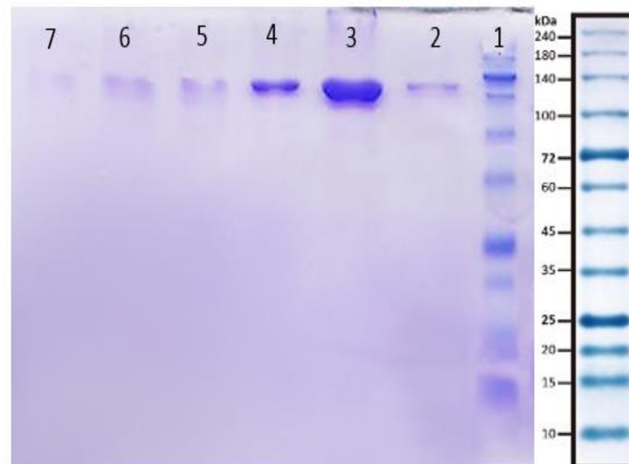

Figure 4. Evaluation of purity of fusion protein in the SDS-PAGE gel; Lane 1: Protein Molecular Weight Marker, Lane 2: Sample rinsing with Imidazole 150 mM, Lane 3 & 4: 250 mM, Lane 5 & 6: 350 mM, Lane 7: 500 mM.

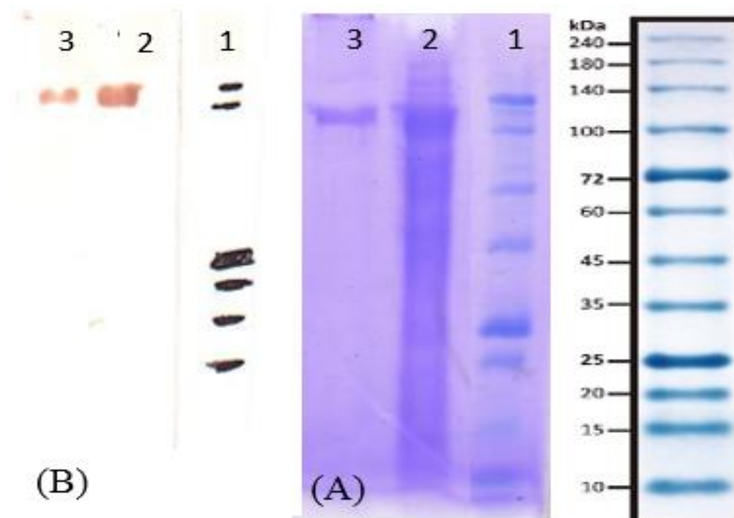

Figure 5. A: Fusion Protein on SDS -PAGE Gel Western Blot; Lane 1: Protein Molecular Weight Marker, Lane 2: Protein Deposition of Induced Recombinant Bacteria, Lane 3: Protein Purified from Recombinant Bacteria. B: Evaluation of antigenicity of the fusion protein with western blotting test; Lane 1: Protein Molecular Weight Marker, Lane 2: Protein Deposition of Induced Recombinant Bacteria, Lane 3: Protein Purified from Recombinant Bacteria.

that encoded by the *arcA* gene[35]. This protein catalyzes hydrolysis of L-arginine to Citrulline and Ammonium[36]. Many studies have been done on the production of fusion/recombinant arginine-deiminase proteins and evaluated its effects[37-40]. Production of a fusion protein, arginine deiminase linked to albumin, along with one or more linker molecules, is one of the recent studies. The results suggest that this combination may be used to treat cancer and Arginine-related diseases in humans and animals, and also as an ingredient in a test kit for the identification of arginine[41].

According to different studies in the field of synthesis, cloning and expression of the azurin and arginine deiminase separately, and the effects of these proteins in the therapeutic and diagnostic fields, we decided to produce the azurin and arginine deiminase proteins from *P. aeruginosa* as a single recombinant fusion protein.

Fusion protein and peptide expression in *E. coli* are confirmed previously. This bacterium efficiently can produce sufficient quantities of protein. Different items should be optimized like the host strain, the chosen vector, growth medium and temperature[42].

In this study, we presented the successful generation and high expression of fusion protein. Cloning and expression was performed in pET28a system under IPTG inducible T7 powerful promoter. The pET28a system is one the most common and suitable systems for cloning and expression of recombinant proteins. On the other hand, in order to obtain high level expression of fusion proteins, *E. coli* BL21 (DE3) was used as an expressed host. *E. coli* BL21 due to its well-known genetics, rapid and inexpensive cultivation is one of the most powerful and diverse expression systems.

Arginine deiminase-azurin fusion protein was expressed in BL21 cells both the concentration of inducer and time of incubation have effect on the expression and production of protein. According to bioinformatics findings, this fusion protein is a stable protein and it can withstand long incubation time. Together with the incubation time, the temperature at which cells are grown is important. The concentration of IPTG and incubation time was optimum at 0.5 mM and 3 hours respectively, and good expression was observed in 37 temperature.

Accuracy in recombinant constructs containing *arcA-azu* with enzymatic double digestion was confirmed. Regards to SDS-PAGE results, recombinant proteins are significantly expressed in cell lysis compared to non-inducible samples. Additionally, we used the C-terminal His tag on the pET28a vector to identify the protein in Western blotting and purify the fusion proteins by Ni-NTA affinity column. Studies have shown that the resin-nickel agarose system with high-affinity and selective used to fusion proteins that tagged with six histidine residues[43]. The procedure for producing fusion protein is quite convenient and efficient and would allow a laboratory to produce large amounts of fusion protein.

The molecular weight of the azurin and arginine deiminase proteins is 62.5 kDa, which together with the linker protein and added sequences, the weight of the synthesized fusion protein reach up to 65.05 kDa. The presence of the induced band in the range of 65 kDa of protein corresponded with the expected molecular weight. Therefore, results showed that the combination of a fusion protein containing azurin and arginine deiminase has been properly prepared and purified; and Western stain testing indicated the reactivity of this fusion protein by the Anti-poly histidine HRP antibody.

Conclusion

In conclusion, our data showed that the *arcA-azu* fusion gene can be expressed by pET28a vector in E.*coli* and we have successfully produced the recombinant arginine deiminase-azurin fusion protein with the presented procedure for preparation of large quantity of fusion protein for structure function studies. According to our data, further investigation could be on evaluation of the anti-cancer properties of this fusion protein and its clinical potential in therapy.

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