ISSN: 0975-766X
CODEN: IJPTFI
Available Online through
Research Article
www.ijptonline.com

ISOLATION AND PURIFICATION OF STAPHYLOCOCCUS AUREUS HIBERNATION-PROMOTING FACTOR INACTIVATING OF THE RIBOSOME

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Received on 08-06-2016 *Accepted on 29-06-2016*

Abstract:

In *Staphylococcus aureus* hibernation-promoting factor (SaHPF) binds to ribosomes depleting translation and turning metabolism of this pathogenic bacteria in energy saving mode. This phenomenon can be recognized as unspecific resistance mechanism, which is exploited by staphylococci to persist in presence of large spectrum of antibiotics. Investigation of structure of SaHPF and its interaction mechanisms with ribosome can aid to design new drugs, rendering *S. aureus* sensitive to antibiotics. We developed techniques for SaHPF purification, which provide this protein in appropriate quantity and quality for further structural analysis. Gene Sahpf has been cloned in expression vector pGS21a. To facilitate purification process His-tag coding sequence was introduced into Sahpf gene to generate SaHPF protein with six histidine residues on C-terminus. *Eschercha coli* strain BL21star (DE3) harboring Sahpf::pGS21a was used for the protein expression. The protein purification was conducted using affinity chromatography with Ni-NTA resin followed by size-exclusion chromatography in Superdex-75. High purification rate and homogeneity of SaHPF preparations obtained allows further study of this protein using nuclear magnetic resonance and development of crystallization procedure for X-ray analysis.

Keywords: hibernation-promoting factor Staphylococcus aureus, ribosome, isolation and purification protein, structure, NMR.

1. Introduction

Gram-positive bacteria *Staphylococcus aureus* is nosocomial infection and causal agent for the variety of diseases such as pneumonia, meningitis, endocarditis and many others [1,2]. Worldwide spread of this pathogen can be explained by large variety of adaptations, one of which is a hibernation of *S. aureus* under unfavorable conditions [1,2]. Hibernation in *S. aureus* is a result of ribosome inactivation by *S. aureus* hibernation promoting factor

(SaHPF), which aggregates ribosomes, forming dimers [3]. The ribosome dimerization allows the cell to sequester energy consumption by translation process, so to survive under unfavorable conditions [4]. Since inactivated ribosomes are not sensitive to variety of antimicrobial compounds, hibernating *S. aureus* can survive during antibiotic therapy.

The ribosome dimers are observed in all growth phases of *S. aureus* and they increase in number when the cell culture transits to stationary phase. Analysis of ribosome dimers revealed that SaHPF binds to 70S ribosome in the molar ratio 1:1, so transition of *S. aureus* to the hibernation requires considerable amounts of SaHPF.

Structural study on SaHPF - ribosome interaction can help in design of new drugs, arresting transition of *S. aureus* cells into hibernation mode, thus making this pathogen sensitive to antibiotics.

Structural analysis performed *in silico* allows to obtain preliminary data on domain folding in protein molecules; molecular dynamics simulation method [5,6] provides a prediction for domain motions. This makes possible comparison of different homologous proteins to reveal common and unique structures in their composition on computer models [7]. Nevertheless, *in silico* approach requires confirmation of structure using Nuclear Magnetic Resonance (NMR) or X-ray crystallography methods, which are considered as credible and accurate methods for protein structure determination.

The aim of the work described here is developing of isolation and purification technique for staphylococcal hibernation protein SaHPF, suitable for NMR or X-ray crystallography study.

2. METHODS

Strains and growth conditions

E. coli strain BL21star (DE3) was grown in LB broth (Difco Laboratories, USA). For solid media preparation 15 grams of agar (Difco Laboratories, USA) was added for one liter of LB broth. Expression vector with insertion of *Sahpf* gene (*Sahpf*::pGS21a) was kindly provided by Dr. Polikanov Y. In this construct structural gene *Sahpf* was fused with six codones of histidine to generate His-tag on C-terminus of expressed SaHPF. Cells of strain BL21star (DE3), harboring *Sahpf*::pGS21a were cultivated in agarized LB medium or LB broth, supplemented with ampicillin (Ap; DiaM, Russia), added to final concentration 100 $\mu g \times ml^{-1}$.

Transformation

Sahpf::pGS21a was transformed into *E. coli* strain BL21star(DE3) using modified $CaCl_2$ method [8]. Cells of strain BL21star (DE3) were plated on LB agar and grown for 15 – 17 hours. One full loop of biomass of strain BL21star

(DE3) was suspended in 1 ml of sterile ice cold 0.1 M $CaCl_2$ solution and incubated for 1 hour in ice. The cell suspension was centrifuged for two minutes at 4000 rpm at 1-4°C. The pellets were resuspended in 100 µl of sterile ice cold 0.1 M $CaCl_2$ solution and incubated for another 20 minutes in ice. *Sahpf*::pGS21a (100 ng) were added to 100 µl of cell suspension mixed and incubated at least 20 minutes. For heat shock the suspension was incubated for 1 minute at 37°C, then immediately transferred back in ice for 2 minutes. One milliliter of LB broth was aseptically added to the cell and the suspension was incubated for 1 hour at 37°C for bacterial cell recovery. The suspension was plated on LB agar supplemented with Ap. Single colonies were inoculated into LB broth amended with Ap.

To test integrity of transformed *Sahpf*::pGS21a in *E. coli* strain BL21star (DE3) plasmid DNA was isolated from liquid cultures on LB with Ap using GeneJet Plasmid Miniprep Kit (Thermo Scientific, Lithuania). Isolated plasmid DNA was hydrolised with NdeI and XhoI (Thermo Scientific, Lithuania) as recommended by manufacturer. Reaction results were checked in electrophoresis in 1% agarose gel in 1xTBE buffer.

Induction of protein expression

Overnight culture of *E. coli* strain BL21star (DE3) harbouring *Sahpf*::pGS21a was grown in LB broth supplemented with Ap at 37°C with aeration. Ten milliliters of the overnight culture were inoculated in 1L of LB broth with Ap and incubated at at 37°C under vigorous agitation 180 rpm. When until culture density reached OD_{600}=0.3-0.4 IPTG was added to final concentration 1мM. The culture with the inductor added was grown for another four hours under the same temperature and aeration conditions. The cell pellets were precipitated at 5000 rpm and rinsed with Resuspending Buffer (RB; pH 7.6) comprising of 20 mM Tris-HCl, 500 mM NH_4Cl. Pellets were frozen and stored at -80°C.

Isolation and purification of protein

The pellets were thawed and cells resuspended in RB amended with DNAse and protease inhibition cocktail. Cells in the suspension were destroyed in Constant Cell Disruption System (Constant Systems Limited, UK). The lysate obtained was cleared using centrifugation at 25000 g and 4ºC for 30 minutes using Avanti 26X centrifuge (Beckman Coulter, Ireland) followed by centrifugation in ultracentrifuge Optima 80XN (Beckman Coulter, Ireland) at 45000 rpm and 4ºC for 45 min.

Cleared lysate was applied on a column, containing Ni-NTA Superflow resin (QIAGEN, USA). To remove non-specifically bound proteins the resin was washed with Saline Buffer (pH 7.6) containing 20 mM Tris-HCl, 1 M NH_4Cl [9] and Washing Buffer with imidazole (pH 7.6) containing 20 mM Tris-HCl, 500 mM NH_4Cl and 20 mM

Imidazole. Target protein was eluted using Elution buffer (20 mM Tris-HCl, 500 mM NH_4Cl, 300 mM Imidazole) pH 7.6. The protein eluted was concentrated using precipitation with ammonium sulfate. Final purification was carried out in column filled with Superdex 75 10/300 in FPLC system AKTApurifier (GE, USA) using gel filtration buffer comprising of 20 mM Tris-HCl, 250 mM NH_4Cl. Purification quality was determined in PAAG disc-electrophoresis 4,5/13,5%. Protein bands were visualized by staining with Coomassie brilliant blue G250. Homogeneity of protein samples were tested using Dynamic Light Scattering (Wyatt Technology, USA).

Solutions (2 µl) with protein concentration 9 mg/ml was applied on crystallization plates and two times diluted in six different commercially available solutions: ProComp[10], Top96[11], Classics[12], Nucleic[13], Salt RX[14], PEG Ion pH[15]. Crystallization process was performed in «Mosquito» system [16] at 4ºC and 20ºC.

3. Results and Discussions

Plasmid *Sahpf*::pGS21a conferred *E. coli* strain BL21star(DE3) resistance to ampicillin to final concentration 100 µg/ml and did not contained rearrengements according to pattern, generated after digestion with NdeI and XhoI. Liquid culture of BL21star(DE3) harboring *Sahpf*::pGS21a yielded five grams of cells.

After cell destruction, protein isolation and purification using affinity chromatography 80 mg of SaHPF with approx. 25% of impurities were obtained from 5 g of cells. Gel-filtration (Fig.1) allowed to obtain 40 mg of SaHPF protein of 95% purity. Protein samples for electrophoretic analysis have been taken on each stage of the isolation procedure. Electrophoretic separation in PAAG demonstrated that SaHPF protein is soluble under given growth conditions and doesn't form inclusion bodies (Fig.2).

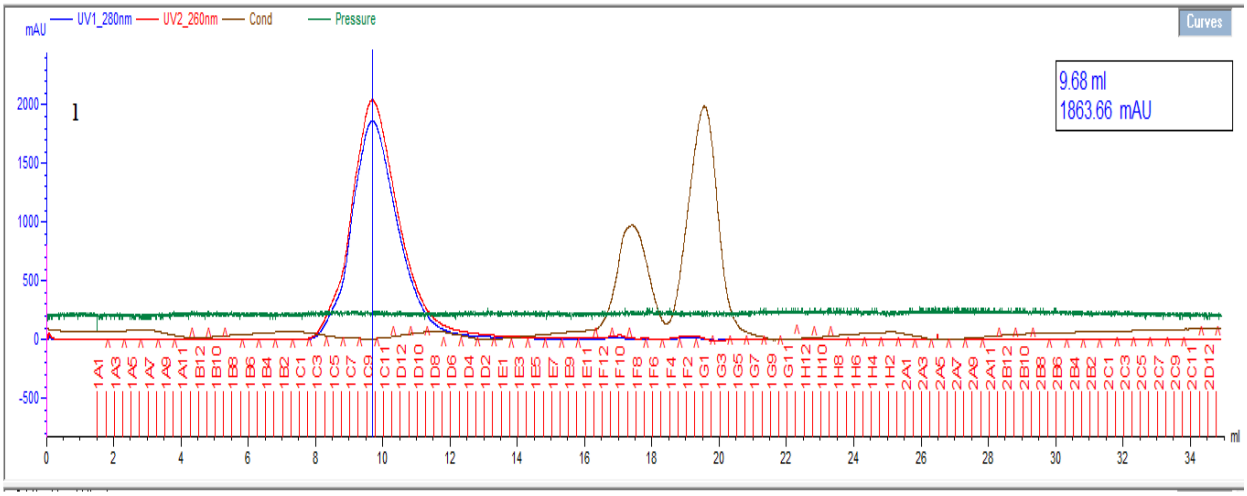

Figure 1. Gel filtration profile for SaHPF purification. First peak corresponds to SaHPF protein, next two peaks belong to imidazole and salts. Blue curve represents adsorption at 280 nm wavelength, red curve shows adsorption at 260 nm, brown curve represents electrical conductivity, green curve represents pressure in chromatography system

during gel-filtration procedure. Y-axis shows adsorption scale at 280 nm wavelength. Numbers of fractions distributed in 96 well plates are given in red above X-axis, which shows volume of buffer passed column in ml. Reading of the buffer volume and absorbance at position, which marked with blue vertical line are given in rectangle in upper right corner of Figure 1.

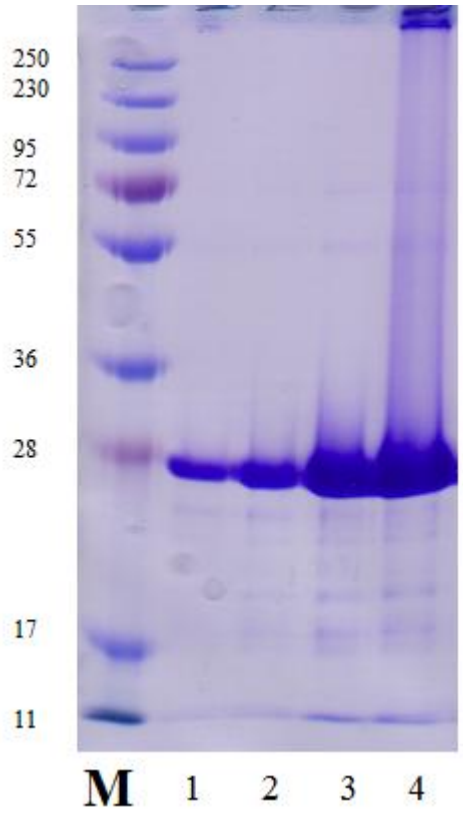

Figure 2. Electrophoresis of SaHPF protein in PAAG; protein SaHPF purified with gel filtration loaded in different amounts. Lines: M – molecular wait markers (protein sizes are given on left side of the gel on the level of corresponding band); 1- 0.8 µg, 2 – 1.6 µg, 3 – 4.1 µg, 4 – 8.3 µg.

Homogeneity of protein samples was estimated using Dynamic Light Scattering method, which showed that solution with SaHPF concentration 9 mg per ml had no aggregations and average molecule radius is 4-4.5 nm.

Commercial solutions which proved their efficacy for crystallization triggering of other proteins were used to stimulate crystal formation of SaHPF protein. Unfortunately, within three month of observation none of the solution used provided conditions for crystal formation by SaHPF. The protein was frequently precipitating in droplets, in several variants the wave was formed. Nevertheless, we continue search of conditions providing SaHPF protein crystallization.

It is known that homologous proteins from other organisms have motile domains. For example in 1IMU protein, which structure was solved using NMR[17], has first domain with stable tertiary structure, but its second domain

motile and existed in several dispositions. There is a possibility that domain motility explains absence of the crystal formation by SaHPF. We propose that structure solving for SaHPF would be more appropriate using NMR method

4. Conclusions

Basic protocol for SaHPF isolation and purification using *Sahpf*:: pGS21a was developed. Being expressed *E. coli* strain BL21(DE3) harboring *Sahpf*:: pGS21a produced 8 mg of the target protein per gram of cells. SaHPF contains his-tag on C-terminus to facilitate purification. The modified SaHPF is soluble.

The technique developed in this work in currently used for structural analysis of SaHPF protein using NMR methods and for crystal formation technique design.

Acknowledgments

The work is performed according to the Russian Government Program of Competitive Growth of Kazan Federal University. The work was funded by RFBR, according to the research project No. 16-34-60001 mol_a_dk., the European Research Council advanced grant 294312. We are grateful to Dr. Polikanov for the plasmid and cells.

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