Iranian Journal of Basic Medical Sciences

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Recombinant production and affinity purification of the *FraC* pore forming toxin using hexa-His tag and *pET* expression cassette

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ARTICLE INFO

Article type:
Original article

Article history:
Received: Jul 2, 2016
Accepted: Jan 12, 2017

Keywords:
Fragaceatoxin C
Recombinant protein
Gene cloning
Hemolysis
Sea Anemone

ABSTRACT

Objective(s): A newly-introduced protein toxin from a sea anemone, namely fragaceatoxin C is a protein with molecular weight of 20 kDa and pore-forming capability against cell membranes has recently grasped great attentions for its function. In this study, its coding sequence cloned as a fusion protein with His-tag for simple production and rapid purification.

Materials and Methods: After PCR amplification using *NcoI* and *HindIII*-harboring primers, the gene fragment was cloned into *pET-28a(+)*. *Escherichia coli BL21* was used for expression of constructed vector and toxin expression was verified by SDS-PAGE. For one-step purification Ni-NTA sepharose affinity chromatography was employed. For examination of purified toxin function, RBC hemolytic test was conducted.

Results: The results showed that the FraC-coding gene was successfully cloned between *NcoI* and *HindIII* restriction sites and purified with affinity chromatography. Densitometric analysis represented the purity of approximately 97%. Hemolytic test indicated the purified FraC had remarkable lytic activity on RBC and almost lysed 50% of cells at the concentration value of 6.25 nM.

Conclusion: The results indicated that not only purified toxin preserved its activity during expression and purification processes but also exerted its function at lower concentrations so that even the 0.09 nM displayed hemolytic effect.

► *Please cite this article as:*
Imani M, Zarei Jaliani H, Kheirandish MH, Azadpour M. Recombinant production and affinity purification of the *FraC* pore forming toxin using hexa-His tag and *pET* expression cassette. Iran J Basic Med Sci 2017; 20:380-385; 10.22038/IJBMS.2017.8577

Introduction

Sea anemones, which belong to the order Actiniaria, are water-dwelling and predatory animals produce quite various protein toxins. Approximately, 32 species of sea anemones have been reported to produce and secrete protein toxins (1). Based on the toxins functions, they are classified into neurotoxins and cytotoxins (cytolysins or actinoporins) (2). The latter ones are group of proteins which exhibit various biological activities including hemolysis and cell cytotoxicity (1, 3, 4). They fulfill this essentially due to their ability to bind cell membranes and form pores which in turn increase cell membrane permeability (5). Any interference in cellular membrane integrity leads to the loss of cell ability in maintaining ions balance across the membrane and hence cell death is unavoidable.

The molecular weights of most-studied cytotoxins are diverse and range between 5-80 kDa. Among them, toxins with almost 20 kDa represent proteins which display pore-forming activity. Molecular cloning and cDNA sequencing have helped identification and characterization of structural and functional characteristics of 20 kDa toxins (6, 7). Due to their pore-forming capability and cell lysis ability, they have been acknowledged as promising agents in controlling tumor cell growth (8, 9).

Several studies have demonstrated that protein toxins such as Pseudomonas exotoxin, diphtheria toxin, and *Clostridium perfringens* enterotoxin are very potent and useful in cancer therapy, therefore they have been considered as promising strategy in cancer treatments studies (10-12). In addition to bacterial protein toxins, sea anemone pore forming toxins have gained a great attention, nowadays (3, 13).

Actinia fragacea is one of the sea anemones which belongs to the genus *Actinia* inhabits the intertidal zone of the northern rocky coast of Spain facing the Cantabrian Sea and the Bay of Biscay. Bearing yellow or green spots on its red column, *A. fragacea* is also known as strawberry (14). It's venom contains a toxin named Fragaceatoxin C (FraC) that

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is a hemolytic toxin cloned and partially characterized by Gonzalez-Manas group in 2009 (4). The cDNA for FraC is available under the accession number FM958450 in GenBank. FraC contains 179 amino acids with relative molecular weight of 20 kDa Moreover, the crystal structure of FraC was resolved in 2010 and deposited in the RCSB PDB under the accession number 3LIM (5).

Structurally, each FraC toxin monomer (protomer) consists of ten β-strands and three helices. In the three-dimensional structure of cytosolic FraC, a β-sandwich core is prominent which is flanked at one side by the β_1-strand, the 3_{10} and α_1 helices at the N terminus, and at the opposite side by the nearly C-terminal α_2 helix (Figure 1A). The α_1 helix is essentially contributed in pore formation. Interestingly, upon interaction with cell membrane FraC undergoes drastic conformational alterations and incorporates its C-terminal α_2 helix into cell membrane (15). The FraC octamer in lipid bilayer have been deposited under the accession number 4TSY, illustrated in Figures 1B, C.

The potential of pore forming toxins in cancer treatment has opened a new area and strategy for cancer research (16, 17). Pore forming toxins are capable proteins and peptides that wide range of organisms produce and utilize as strong defense biological weapons (18). Moreover, intensive attention has been directed toward the production of recombinant form of anticancer protein agents for medical purposes (19-21).

Due to potential applications and importance of pore forming toxins in cancer treatment and structural analysis the aim of the present study was to introduce a simple production approach by cloning of FraC as a fusion protein with His tag in *pET* expression vector, and to express in *Escherichia coli*, to purify and to evaluate its biological activity against red blood cells.

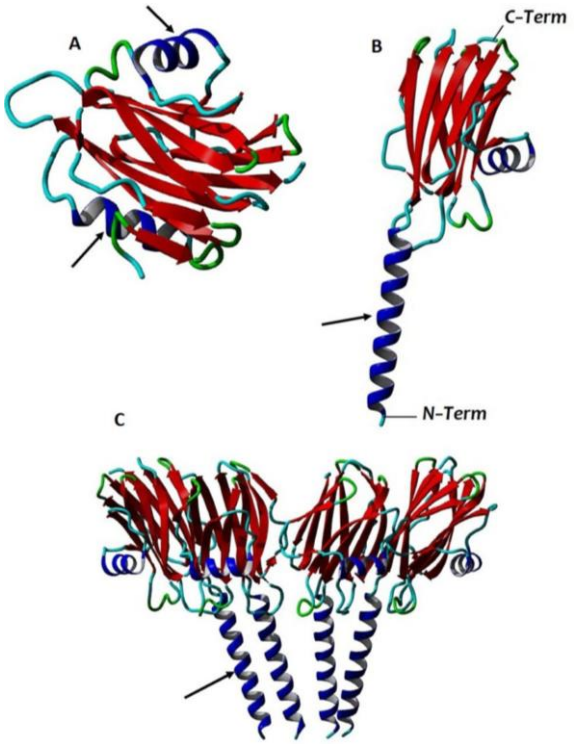

Figure 1. Crystal structure of different forms of FraC. (A) water soluble and cytosolic conformation of FraC in which two distinct α-helices are indicated by dark arrows. (B, C) Altered structure of FraC in lipid bilayer. (B) A monomer of pore complex and (C) half structure of pore complex. All 3-D structures were created by YASARA view software

Materials and Methods

NcoI, *HindIII*, *T4* DNA ligase, isopropyl-b-D-thiogalactopyranoside (*IPTG*), and *Pfu* DNA polymerase were purchased from Fermentas. Polymerase chain reactions (PCR) reagents, DNA ladder (SL5051) were obtained from Sinaclon Co. Plasmid extraction kit, PCR purification kit, were obtained from Bioneer Corp. Ni-NTA (nitrilotriacetate) spin kit was from Novagen Inc. Tryptone, yeast extract, and agar were obtained from Scharlau company. All SDS-PAGE chemicals were purchased from Merck. Ampicillin and kanamycin were from Sigma-Aldrich. Protein Ladder (PR901641) was from CinnaGen Co.

Microorganisms and plasmids

pBAT-4 containing FraC gene was a kind gift from Prof Dr Gonzalez-Manas. *pET-28a(+)*, *E. coli DH5α, E. coli BL21 (DE3) pLysS* cells were obtained from Pasteur institute of Iran.

The *pET-28a(+)* is a powerful system developed for cloning and high-level expression of recombinant proteins in *E. coli*. Gene of interest is cloned under the control of a strong bacteriophage T7 promoter which its expression is induced by IPTG or lactose. It bears one thrombin protease digestion site and two His Tag sequence at either side of multiple cloning site. Moreover, carrying kanamycin resistance gene sequence allows investigators to screen transformed bacterial cells by growing them on kana medium.

Visualization of 3D structure of FraC

The crystal structure illustrations presented here were generated by user-friendly YASARA view software (http://www.yasara.org).

Amplification of FraC gene

For amplification of FraC coding sequence, 50 ng of the plasmid containing cDNA of FraC was used as a template. The PCR was carried out by *Pfu* DNA polymerase under the following conditions: the initial denaturation 94 °C for 5 min, 30 cycles (94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min), and the final extension was implemented for 10 min at 72 °C. The following synthetic oligonucleotides were used for amplification: Forward primer 5' GGAGGGAT***CCATGG***

CCGATGTTGCAGGTG 3' and reverse primer 5'GCGG-***AAGCTT***AGCCTTGGTCACGTGAATTTCCAG 3'. The primer pair contains restriction sites for *NcoI* and *Hind* III, (underlined sequence) for cloning into the bacterial expression vector *pET-28a(+)* in frame followed with nucleotides encoding a 6-histidine tag sequence which is already available on vector body.

Construction of expression vector

Once FraC gene amplified, the PCR product was loaded on the 1% agarose gel and the expected band (540 neuclotide) cut out from the gel and purified using DNA gel extraction kit and went under digestion with two restriction enzymes *NcoI* and *HindIII*. After purification of digested insert by clean up kit, ligation reaction started by mixing digested FraC gene with digested *pET-28a(+)* in the ratio of 10:1 and T4 DNA ligase at 24 °C for 2 hr. Inactivation of T4 DNA ligase was performed at 70 for 20 min. The resulting construct named *pET-28a(+)-FraC*.

Transformation

Five µl of ligation mixture was added into 50 µl of *E. coli DH5α* competent cells, incubated on ice for 30 min, heat shocked for 90 sec at 42 °C and incubated again on ice for 10 min. Then, 1 ml of SOC media was added to the tube and incubated for 1 h at 37 °C followed by streaking cell pellets on LB agar-plates containing 50 µg/ml of kanamycin and incubated at 37 °C overnight (O/N).

Confirmation of cloning

Following heat-shock transformation of ligation product into *E. coli* DH5α competent cells and growing them on kana agar plates at 37 °C O/N, five positive bacterial colonies randomly picked up from agar plates, grown in LB medium, and their plasmids was extracted by plasmid miniprep kit. The purified plasmid used as templates for colony PCR using FraC-specific oligonucleotides as primers. Finally, *pET-28a(+)-FraC* constructs were sequenced using an automated sequencer (MWG) using T7 promoter and T7 terminator universal primers.

FraC Protein induction and purification

A single and fresh bacterial colony harboring the expression plasmid *pET-28a(+)-FraC* was used to inoculate 10 ml of LB medium containing 50 µg/ml of kanamycin. The culture was then incubated at 37 °C with vigorous shaking O/N. Next, 200 ml of LB media was inoculated with 2 ml of O/N culture and grown at 37 °C with vigorous shaking until the OD of culture density reached 0.6. IPTG was added to the final concentration of 1 mM and the culture grown at 22 °C for 5 hr. Bacteria expressing FraC were collected and resuspended in lysis buffer (phosphate buffer, pH 7.4 (contained 300 mM NaCl and 5 mM imidazole), and sonicated in ice water bath. The lysate was centrifuged at 4 °C for 20 min at 10000 g. Purification of His6-tagged fusion FraC was carried out using Ni-NTA spin column according to manufacturer's instructions. Briefly, the column was first washed with 10 ml of distilled water and then equilibrated with 10 ml of lysis buffer. The cell supernatant was loaded on a Ni-NTA column. Then, the column was washed with 20 ml of wash buffer (the same as lysis buffer except that of 25 mM imidazole). After that, the recombinant FraC was eluted using elution buffer (Phosphate buffer containing 300 mM imidazole). Finally, the purity of the FraC protein was analyzed by SDS-PAGE stained with Coomassie Brilliant Blue. Protein concentration was determined by Bradford assay using BSA as a standard (22). in order to estimate the purity of toxin on PAGE densitometric analysis Totallab CLIQS 1D software was used.

Examination of FraC activity

In order to examine the activity of purified FraC, hemolytic test was performed according to Bellomio *et al* with a little modification (4). Briefly, sheep red blood cells were washed thrice with PBS, pH 8. RBCs were resuspended in PBS and the Absorbance at 700 nm was adjusted to around 1. Hemolytic assay was performed by recording the changes in turbidity (A_{700}) in a Unico UV2100 spectrophotometer after a 15 min incubation of RBC solution with various concentration of the purified toxin. Technically, the phosphate buffer in which the FraC dissolved was used as blank. Next, the A700 of intact RBC solution adjusted near 1 and eventually reduction in absorbance of the toxin-treated samples were measured. Hemolytic activity is expressed in percent using the following formula:

$$H\ (\%) = (A_{max}-A_{obs})/(A_{max}-A_{min})\times 100,$$

Where the A_{max} and the A_{min} represents the absorbance of the intact and completely lysed RBC respectively. A_{obs} is the absorbance of the each sample recorded after 15 min incubation with each concentration of the toxin.

Results

Amplification of FraC gene

In order to clone the FraC gene into *pET-28a(+), pBAT-4* harboring FraC used as a template for PCR amplification. As shown in Figure 2 the resulting amplified fragment was 540 nucleotides length.

Construction of pET-28a(+)-FraC construct

In the next step of cloning, to clone amplified FraC gene into *pET-28a(+)* expression vector, the vector is digested by two restriction enzymes *NcoI* and *HindIII*. The restriction sites of *NcoI* and *HindIII* were chosen for cloning of FraC so that the N-termianl His-tag and thrombin protease digestion site were removed and only the 6His-tag would be fused in C-termius of the FraC protein which would help for one step purification.

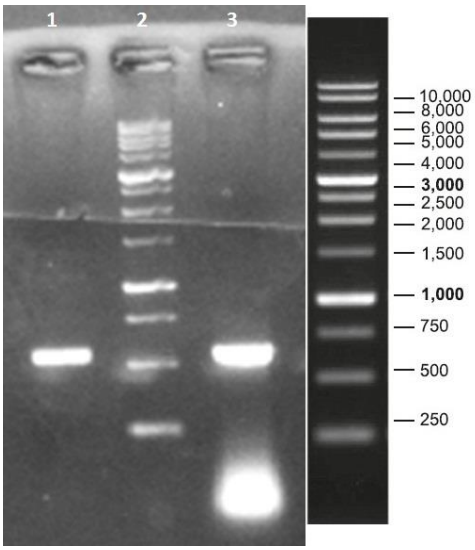

Figure 2. PCR product of FraC. The lane 1 and 3 represents two samples of amplified fragments of FraC which correspond to 540 nucleotide. The lane 2 shows 1 kb DNA ladder

The undigested and digested *pET-28a(+)* are shown in the Figure 3. As Figure represents, the digested vector is in the linearized form which nearly corresponds to 5231 bp band after excluding 138 bp between two above-mentioned restriction enzymes sites.

Confirmation of cloning

In order to confirm the insertion of FraC into *pET-28a(+)*, two methods were used. First by the help of colony PCR it is verified that four out of five colonies were positive for FraC gene insertion. As the Figure 4 indicates only the colony three lacks the FraC gene and all the rest colonies absorbed the full construct. For final confirmation and correctness of FraC cloning in frame, sequencing of constructs was performed with T7 promoter and terminator universal primers (data not shown).

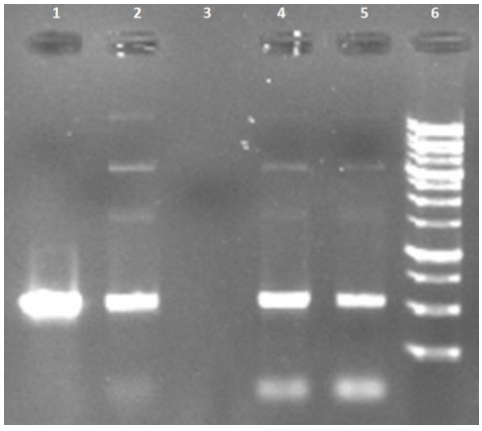

Figure 4. Colony PCR of positive colonies. Lane 1 to 5 are the PCR of colonies. As apparent in the Figure, only the colony 3 is negative and no amplification happened. Lane 6 is 1 kb DNA ladder. The molecular size of DNA ladder used in each gel represented at the right side of Figure 2. The percentage of agarose gel is 1%

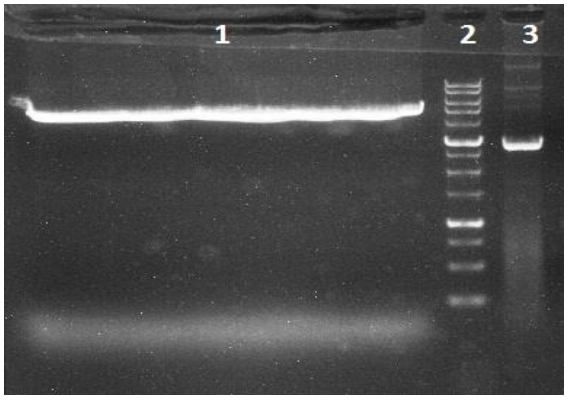

Figure 3. The lane 1 shows the digested vector with enzymes *NcoI* and *XhoI* for gel extraction. Lane 3 displays intact minipreped vector in various conformation. Lane 2 is 1 kb DNA ladder. The blurry band in the lane 1 is the 139 nucleotide separated from the vector after restriction digestion. The molecular size of DNA ladder used in each gel represented at the right side of Figure 2

Expression and purification

The expression of FraC was induced with 1 mM IPTG. SDS-PAGE analysis showed a high degree of purity following purification by Ni^{+2}-NTA affinity chromatography. The purified toxins are apparent in lanes 2 and 4 of the gel (Figure 5).

Hemolytic activity

Biological activity of purified FraC was assessed and depicted in Figure 6. The concentration of purified FraC determined 0.40 mg/ml by Bradford protein assay. Considering molecular weight of 20 kDa for FraC, the molar concentration of 0.40 mg/ml calculated 20 µM. Apparently, the protein destroyed almost 100% of RBC cells by the concentration of 50 nM which followed by a smooth drop in hemolysis in concentration 25 nM. In the concentration of 6.25 nM, 50% of cells were killed. The picture demonstrates the dose-dependency of FraC activity.

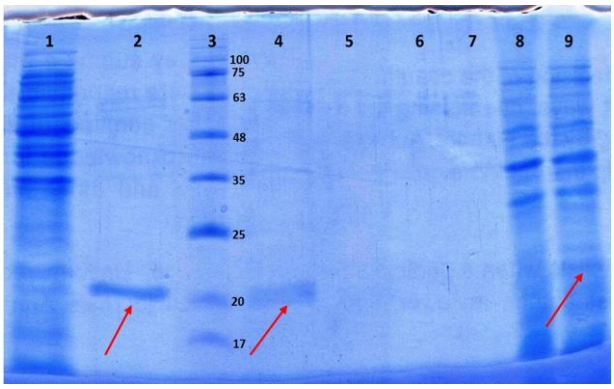

Figure 5. Verification of FraC expression and purification by SDS-PAGE. Lane 1: flow through of the column during washing step; lanes 2 and 4: two successive fractions of purified FraC toxin; lane 3: protein marker; lanes 5-7: fractions contain no protein; lanes 8 and 9: *E. coli* bacteria lysate before and after induction with IPTG 1 mM, respectively

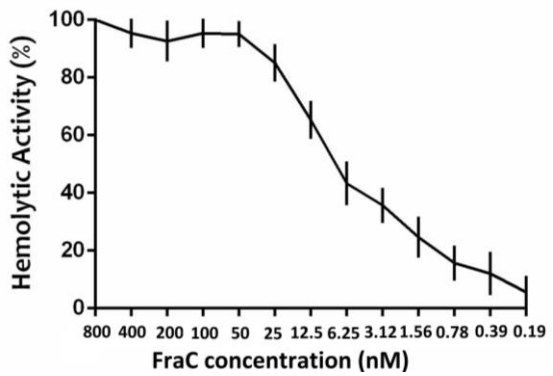

Figure 6. Hemolytic activity of FraC. After purification, various concentration of FraC were prepared and examined on RBS solution which were recorded after 15 min of incubation at 700 nm. As apparent in the Figure the concentration of 6.25 nM approximately destroyed 50% of cells

Discussion

One of the most effective pore-forming toxins which is investigated on cancer cells is equinatoxin from sea anemone (13, 17, 23). The mechanism by which cytolysins exert their toxicity originate from their structural conformation that switch from stable water-soluble state to integral membrane pore encountering cell membrane (18).

Furthermore, for studying structure and function of a protein require a large and sufficient amount of protein. By the help of cloning and recombinant production of proteins using *pET* system and *E. coli* as a host, one can obtain large amount of protein comparing traditional protein purification methods. The aim of this study was to construct and present a simple method for production and purification of sufficient amount of FraC so that it can preserve its activity through purification process and utilized for further studies.

To do so, the cloning of FraC gene in *pET* expression system specifically *pET-28a(+)* was performed. FraC was not cloned as a fusion protein till now, therefore its purification demands a sophisticated purification steps which in turn diminish its yield of purification. To overcome this difficulty, for the first time we successfully cloned, expressed and purified it. To do it, we designed specific primers for amplification of FraC gene which carry *NcoI* and *HindIII* site of digestion at their 5'. The PCR product, indicated that the primers specifically could bind and amplify the FraC (Figure 2). Moreover, ligation of FraC with digested *pET-28a(+)* was achieved at 24 °C for 5 hr. At the last step of cloning, DNA sequencing of construct *pET-28a(+)-FraC* confirmed the gene inserted correctly in frame under the T7 promoter.

Protein expression of FraC was induced by IPTG. As Figure. 5 indicates, the protein was successfully overexpressed after 5 hr at 22 °C. Unlike purification process of non-recombinant proteins which needs multi-step purification methods such as both ion exchange and gel filtration chromatography, by expression of constructed recombinant gene we were able to purify it by affinity chromatography with the help of Ni-NTA column in one step. Comparing the yield of FraC purification by Bellomio *et al* which totally was 0.5 mg per liter of bacterial culture (4), however we purified 0.8 mg from 200 ml culture or 4 g per liter. It means that expression of FraC as a fusion protein resulted in a eight fold increase in the yield of it compared to that obtained when expressed as non-fusion protein.

Densitometric analysis using Totallab software exhibited that the purification of recombinant FraC was successfully carried out by the purity of over 97% (data not shown). Expression and purification of a recombinant protein does not necessarily infer that the protein preserves its conformational structure and hence activity through purification steps. To reveal that the purified FraC maintained its structure and hence its function, we investigated its pore-forming activity against RBC cells. As Figure 6 indicates the FraC exerts it hemolytic activity even at lower concentrations (0.09 nM) which implies its effectiveness. Calculating the FraC concentration that lyses 50% of RBCs we obtained 6.25 nM value which is in consistent with previous study conducted by Bellomio *et al* (4). However, the minimum FraC concentration below which no RBC cell lysis occurred was estimated 0.09 nM while in the Bellomio *et al* work it was 0.8 nM. This implies that our purified FraC is much more effective than the non-fusion recombinant toxin.

Conclusion

In summary, utilizing His tag at the C-terminus of recombinant FraC, we successfully applied one-step purification method using affinity chromatography for production of functional and active FraC. Furthermore, our results showed that, using *pET* system, it is possible to produce FraC in large quantity for further cell biology investigations and biochemical clarifications. Successful production and purification of FraC will help researchers to obtain sufficient amount of FraC for studying its pore forming activity against various eukaryotic cells. Investigation of FraC activity either in the presence of other proteins or lipid structures or under various conditions is highly suggested, as well.

Acknowledgment

The authors would like to thank the Iran National Science Foundation (INSF) for the financial support of this work under the contract number 92000367. Technical support from Mr Ali Pirnejad from the central lab of Faculty of Veterinary Medicine of Urmia University is highly appreciated, as well.

Conflict of Interest

The authors report no conflicts of interest.

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