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Original Article

Cloning and Expression of N-CFTX-1 Antigen from *Chironex fleckeri* in *Escherichia coli* and Determination of Immunogenicity in Mice

Hossein JAFARI [1], ***Saeid TAMADONI JAHROMI*** [2], ***Jamil ZARGAN*** [1], ***Ehsan ZAMANI*** [1], ****Reza RANJBAR*** [3], ****Hossein HONARI*** [1]

1. *Department of Biology, Faculty of Basic Sciences, Imam Hossein Comprehensive University, Tehran, Iran*
2. *Persian Gulf and Oman Sea Ecology Research Center, Iranian Fisheries Sciences Research Institute, Agricultural Research Education and Extension Organization (AREEO), Bandar Abbas, Iran*
3. *Molecular Biology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran*

***Corresponding Authors:** Email: honari.hosein@gmail.com; ranjbarre@gmail.com

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Abstract

Background: Most jellyfish species are poisonous. Human victims of jellyfish sting each year are 120 million. *Chironex fleckeri* is a venomous box jellyfish that inflicts painful and potentially fatal stings to humans. The CfTX-1 is one of the antigenic proteins of venom that is suggested to stimulate the immune system for treatment and vaccine. This study aimed to clone and express the CfTX-1 antigen in *E. coli* and then to determine the synthesis of related antibody in the mice.

Methods: The study was performed in the Persian Gulf and Oman Sea Ecology Research Center, Bandar Abbas, Iran in autumn 2016. The synthetic CfTX-1 gene in PUC57 plasmid was purchased from Nedaye Fan Company. The 723 bp fragment of N-CfTX-1 was amplified by PCR, PUC57 plasmid containing CfTX-1 with BamHI SalI restriction enzyme sites were subcloned in pET28a [+] expression vector and transformed into *E. coli* BL21 (DE3). The CfTX-1 gene expression was induced by IPTG. Then antibody produced from the mice serum were isolated and confirmed by ELISA. After protein purification, resulted antigen was injected to mice in 4 repeats and then evaluated the rate of antibody in mice serum. Mice were challenged by the *Carybdea alata*.

Results: The 726 bp of N-CfTX-1 were cloned in a vector of expression pET28a [+] and confirmed by PCR, sequencing and enzymatic analysis. Moreover, the recombinant protein was confirmed by SDS-PAGE and Western blotting. Then the antibody was isolated from mice serum and confirmed by ELISA test. The results showed that immunized mice tolerated 50x $LD50^1$ of jellyfish venom.

Conclusion: The CfTX-1 recombinant protein was able to protect the BALB/c mice against jellyfish venom. The produced protein can be used as a candidate for vaccine against jellyfish venom.

Keywords: *Chironex fleckeri*; *Carybdea alata*; Venom

Introduction

Jellyfish belongs to the largest known medusa in the Cnidaria phylum and exists extensively in the Persian Gulf and the Oman Sea. The blooms of Jelly fishes become very large in many seasons of the year (1). Most of the jellyfish species are toxic and more than 120 million people are injured by these organisms annually. Although most of these injuries do not require supplementary therapies,

sometimes death occurs (1). The venom of these organisms has a vascular active ingredient such as histamine and chitin that instantly causes severe pain, edema, hives, and sometimes gastrointestinal disorders, muscle spasm and respiratory distress, and cardiovascular failure (2).

The Box jellyfish is the most poisonous species of jellyfish. In the study of compounds of *Chironex fleckeri* venom, more than 61 proteins had a molecular weight between 10 and 180 kDa. Two main proteins by the name of CfTX-1 and CfTX-2 are particular importance. Biochemical studies show that separation of these two proteins is difficult with separation methods such as electrophoresis and chromatography; because these are usually separated by sticking together (3). Obtaining comprehensive information from Cnidaria venom is limited, due to the difficulty and technical difficulties in extracting and storing **venom** and its unstable and sensitive nature. From two main venom proteins, the CfTX-1 protein has about 43 kDa molecular weight and CfTX-2 with 45 kDa. The CfTX-1 protein with the Accession number of AFQ00676.1 has 454 amino acids and CfTX-2 with the Accession number of AFQ00677.1 has 461 amino acids (3). These two proteins are potent antigenic and able to stimulate the immune system (4). These proteins in 379-451 region of amino acid of CfTX-1 and 386-458 amino acid of CfTX-2 have more than 95 percent homology with each other, so, the measurement of quantitative and qualitative of jellyfish venom by ELISA and Western blot methods is difficult. In this research, the genome region of 723 base pairs from N-terminal region of CfTX-1 was selected. These proteins have less than 53 percent homology in the region of 29-240 amino acid of CfTX-1 and region of 35-272 amino acid of CfTX-2 with each other.

By selection of this region and cloning and antigen expression in *E. coli* and antibody-producing and immunization of that in mouse, the quantitative and qualitative measurement of jellyfish will be possible with ELISA and Western blot methods (5). Whereas the box jellyfish venom can cause death by effects on heart, and generally the death occurs shortly after biting, so, the designing and using the vaccine is important for preventing death.

The aim of this study was to clone and express the CfTX-1 antigen in *E. coli* and then to determine the synthesis of related antibody in the mice.

Materials and Methods

Production of recombinant protein

The study was performed in the Persian Gulf and Oman Sea Ecology Research, Center Bandar Abbas, Iran in Autumn 2016. The sequence of CfTX-1 gene of *C. fleckeriIii* was extracted from NCBI gene bank and was sent to the NedayeFan Company for producing it. Plasmid PUC57 was prepared by a gene synthesizing company and the corresponding bacterium was obtained from the laboratory of the Department of Biology for transformation. Ni-NTA agarose resin column was used to purify the recombinant protein purchased from QIAGENE Company.

A) Selection of the desired gene and optimization of synthetic gene sequences

The 723 base pair of gene fragments from N-terminal region of CfTX-1 was selected. This sequence was referred to genscript website to optimize the correct use of the codons for the desired host, correct the content of the GC content, establish an appropriate secondary structure for the mRNA, correct the splicing regions and modify the cleaving of a target site, to avoid interference with cloning. The desired gene was synthesized and cloned by considering the BamHI and Sall digesting sites on PUC57 clone and the recombinant plasmid purified from one of the clones was sent as a dry form.

B) Primer designing of N-CfTX-1 synthetic gene

To have the maximum effect of the BamH1 restriction Enzyme, the leader sequence was designed based on the cut site area of BamH1 restriction enzyme and also to prohibit the overlapping oligonucleotide sequences.

BamHI restriction enzyme recognizes G^GATCC sites which in this case we were designed and prepare the forward primer (AGGATCCGATGTTGATGAAGTGACGAGC3') for this purpose.
Reverse primer sequences were designed based on sequences of SalI restriction enzyme.
The reverse primer is:
CfTX-1 REV5'-CTCGAGTTAGTCGACGATGCTCACAGCATTGGCAT3'

Amplifying of N-CfTX-1 gene fragments with PCR reaction

PCR reaction was done by amplification of gene fragment of 723 base pair from N-terminal region of CfTX-1 and using the pfu polymerase enzyme (Fermentas Company) in 25 µl volume. Each PCR reaction was consist of 0.4 pmol of each primer, 0.4 mM dNTP, 0.25 unit of pfu DNA polymerase enzyme.2.5 µl 10X PCR Buffer and MgSO4 with total volume of 2.5 mM and 50 ng PUC 57 vector. PCR cycles consisted of one primary denaturation step at 94 °C for 45 Sec. Primer annealing in 60 °C for 55 sec and extension of the desired fragment in 72 °C for 60 sec and final extension was 72 °C for 50 minutes.

Preparing the digested Fragments of PCR Product and plasmid vector

For cloning the digested enzyme reaction with BamHI and SalI on the N- terminal of CfTX-1 fragment gene amplified and pET-28a (+) vector was also done for cloning. After the enzyme digestion, the digested CfTX-1 gene fragment and pET-28a (+) were extracted with DNA purification Kit from agarose gel.

Insertion and Transformation

The N-CfTX-1 gene which was cleavage with the *BamH1* and *Sa1I* restriction enzyme and inserted in pET-28a [+] vector which was cleavage also with these enzymes and extracted, too. The insertion process was 60 min at 22 °C which was complete by T4 DNA Ligase enzyme (Fermentas Company). The insertion product was transgenic by thermal shock method of *E. coli* BL21 strain DE3 into susceptible cells (prepared by chemical method).
Recombinant colonies were isolated with kanamycin antibiotic screening, and the presence of plasmid containing CfTX-1 was confirmed by PCR, sequencing and enzymatic digestion.

Expression and purification of CfTX-1 gene

To express the N-CfTX-1 gene, 100 µl from overnight cultures, were inseminated to 5 ml of LB medium containing kanamycin, and after reaching OD at 600 nm (to obtain bacterial growth), The promoter inducing (IPTG) from Fermentase was added to the medium at a concentration of 1 mM and incubated at 37 °C for 4 hours (6,7).

Treatment of bacterial cells collected for studying the expression by electrophoresis

The bacterial cells collected from the culture were treated with denaturation method. In this method, the cells were mixed with 100 µl of buffer B and disrupted by sonication method. The samples were then centrifuged at 14000 rpm for 20 min and the supernatant was blended with a ratio of 1 (sample buffer) to 5 (specimens) with a mixing sample of 5 × 5 concentrations and boiled at 100 °C for 5 minutes. Finally, the samples treated with gel electrophoresis (SDS-PAGE) were investigated in terms of expression of recombinant proteins (8).

Studying the expressed protein by Acrylamide electrophoresis

To prepare the gel solution, the percentage of acrylamide and bis-acrylamide was selected proportional to the protein size. Overall, 12% SDS-PAGE protein gel was used. The expressed protein was about 24 kDa the samples were electrophoresed before and after induction of IPTG, along with a protein marker (synagene) under denatured conditions. The concentration of the gel was 12% with a constant flow of 25 mA. To observe protein bands, staining with ComasiBlue was used (9).

Confirmation of expressed protein with western blotting technique

It was used western blot method to confirm the protein expression. The western blot technique was used to confirm the recombinant protein. Blotting of samples was done on nitrocellulose paper. At first, the 20µg of pure target protein was run in 12% polyacrylamide electrophoresis and BSA protein was used as the as a negative control as the same amount of the target protein, gel was then isolated from the electrophoresis caste and washed in a blotting buffer containing 25 mM Tris, 192 mM glycine and 20% ethanol. After washing, the gel was packed in western blot sandwich and placed in a western tank. The blotting process was performed at 100 mV for 1 hour and a half. To fill the vacant positions (blocking), the paper was kept at 4 °C for 3 nights in a PBST solution overnight. Then it was washed three times with PBST. The paper was heat-treated with mouse poly-clonal antibodies at a dilution of 1: 2000. The washing process was performed with PBST three times. Mouse conjugated with dilution 1: 2000 was used as a diagnostic antibody. As in the previous step, the heating was done. The washing process was carried out as before. Finally, nitrocellulose paper was placed in a dye substrate solution, (60 mg of DAB in 100 ml Buffer, 50 mM Tris buffer with pH 8) until a protein band emerged. To stop the reaction, the paper was placed in distilled water (9,10).

Application of the Ni-NTA column to purify the protein by denaturation method

All purification steps were performed at 4 °C in a cold room and collected protein was stored immediately at -20 °C. The amount of 25 µl of column output was pick up after sampling of the buffer by SDS-PAGE electrophoresis gel for studding goals (9).

Determination of Protein Concentration

Determination of expressed Protein concentration was done by Bradford method using the Bovine Serum Albumin (BSA) from Sinagen Company as standard (11).

Elimination of urea from recombinant protein

In order to use purified protein for immunization, since it is used the urea in purification process, the dialysis method with using a concentration gradient to remove urea, was used to retrieve the recombinant protein as much as possible. At first, in 4 M urea, then in 2 M urea, and then in 1 M urea, and finally, dialysis was done, two times in PBS.

Injection of CfTX-1 recombinant protein into mice and determination of antibody titer by indirect ELISA method

To evaluate the immune response, 5 mice were used as test and 5 were used as control. For each mouse, 20 µg purified protein was fed to a volume of 500 µl with sterile PBS. To prepare the samples for injection, the same volume of the adjuvant, oil was added in water (final volume of 1 ml) and then the contents were homogeneous. Finally, 200 µl of each prepared specimen containing 20 µg of the desired antigen were injected intravascular and then indirect ELISA was used to determine the antibody titer in the animal serum (12, 13).

The challenging of safe animals by jellyfish venom

After immunization of animals, 10, 30 and 50 LD50 of *Carybdea alata* venom were injected to the mouse and the results were studied after 2.5 d and the animals were monitored up to 30 days.

Results

Amplification of CfTX-1 gene

After amplification of the N-CfTX gene by PCR, the PCR product was evaluated on the agarose gel (Fig. 1), and the component was considered to be compatible with the target gene in terms of size (723 bp).

Cloning of CFTX-1 gene in a pET-28 [+] vector

To cloning, the amplified fragment and the expression plasmid pET-28a (+) were linearized with two-enzyme digestion by restriction enzymes SalI and BamHI.

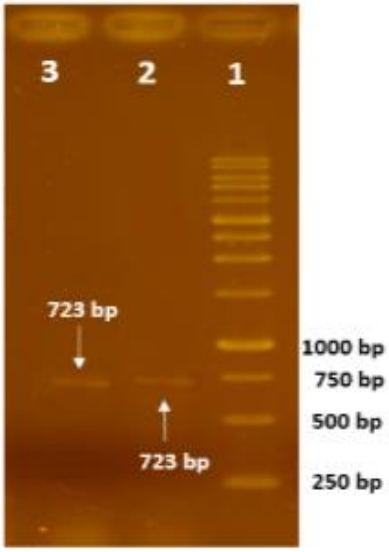

Fig. 1: PCR product. Well 1: molecular nucleic acid marker and wells 2 and 3: the PCR product samples

The desired fragments were purified by using a kit. The gene was obtained with a sequence length of about 723 bp and a plasmid pET-28a (+) with a sequence length of about 5369 bp (Fig. 2).

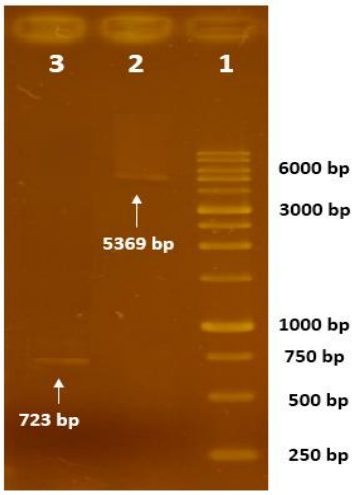

Fig. 2: Plasma pET-28a (+) dual digestion. Well 1: DNA marker. 1 kbp well 2: The band is about 5369 bp. Well 3: The band is about 723 bp

Screening

To confirm cloning, the white colonies were cultured and plasmid was extracted by alkaline lysis method. For plasmids, PCR and digested restriction enzyme were laid. After amplification of the CfTX-1 gene by PCR, the PCR product was evaluated on a 1% agarose gel and the target portion (723 bp) consistently matched to our target gene (Fig. 3a). To confirm the enzyme digestion, after purification of the plasmid and confirmation with 1% agarose gel, the plasmids were digested with two BamHI and SalI restricted enzymes. Then, with the help of the marker, the size of the extracted fragments of the vector (723 bp and 5369 bp) was confirmed (Fig. 3b).

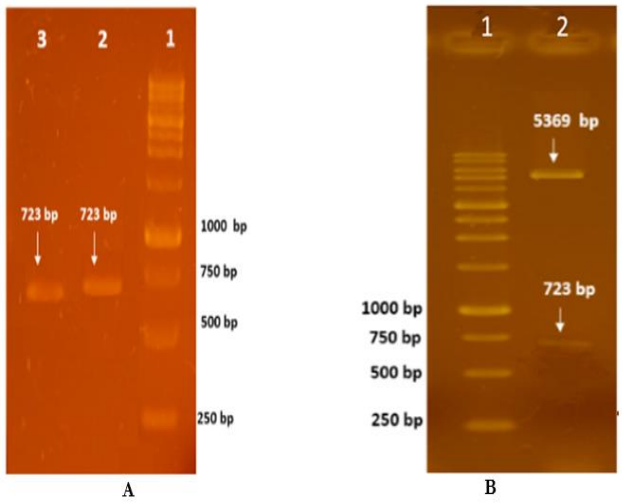

Fig. 3: Confirmation of target gene cloning in pET-28a (+) A] Wells 1: Nucleic acid Marker - Wells 2 and 3: PCR product on pET-28a plasmid (+). B] wells 1: Nucleic acid marker 2: The enzyme digested product on pET-28a (+)

Investigation of CfTX-1 gene expression in pET28a vector [+]

At this stage, after cell culture and induction with IPTG, expression of the gene was performed and analyzed by SDS-PAGE gel after treatment. Regarding the weight of the recombinant protein, SDS-PAGE protein gel was used (Fig. 4).

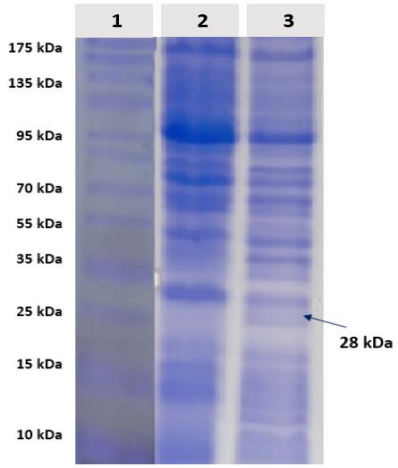

Fig. 4: SDS-PAGE gel electrophoresis to determine the expression of the desired protein. Column 1: Protein Marker, Column 2: Negative control in which bacteria do not induce IPTG after reaching optical absorption of 0.6 at a wavelength of 600 nm (B Lysis buffer), Column 3: Protein content of bacteria-induced by IPTG after reaching their absorbance of 0.6 at 600 nm (B Buffer).

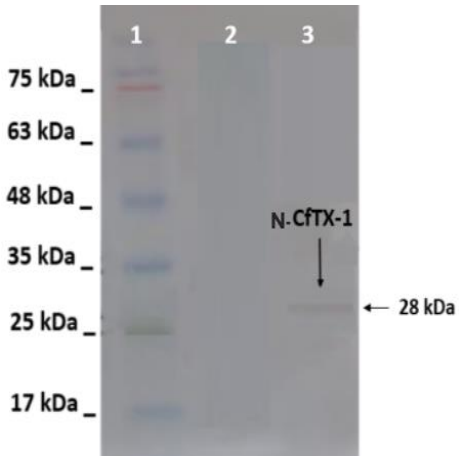

Fig. 5: Confirmation of recombinant protein expressed by western blot method using polyclonal antibody of jellyfish venom. Column 1: Molecular Marker 310003, Column 2: Control without IPTG Induction, Column 3: Specimen of the desired Protein Band.

Confirmation of Protein Product obtained with Western Blotting Technique

In the test column, related to the IPTG-induced sample, there is a band near kDa28. In the control column that was not induced by IPTG was not seen (Fig. 5).

Evaluation of antibody against recombinant protein by indirect ELISA

After each injection, the antibody titer increased compared to the control, and in the last bloodletting, the highest amount of antibody against the recombinant antigen was created. The average of the antibody titer in each stage is shown in Fig. 6.

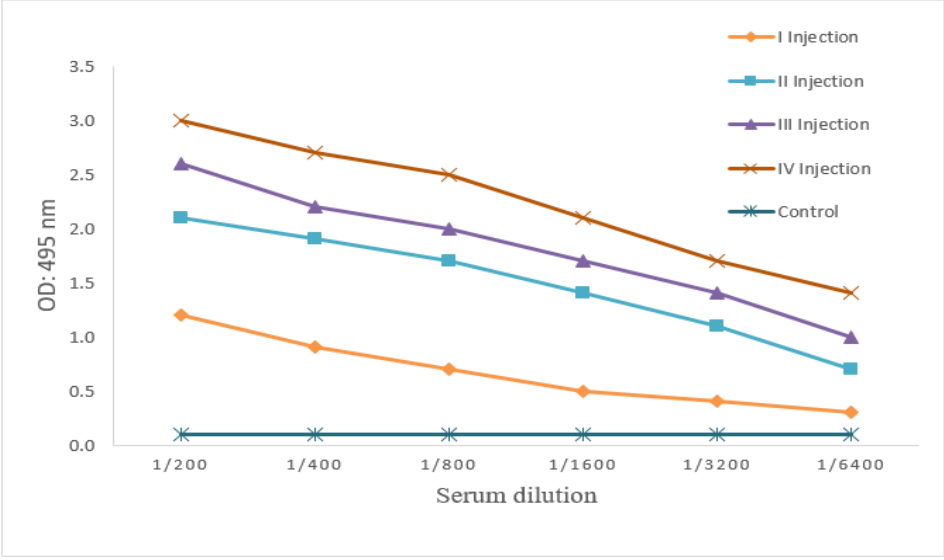

Fig. 6: Evaluation of BALB/c antibody titer after each injection with ELISA

The challenge of BALB/c mice with jellyfish venom

After the mice were immunized and challenged with 50 LD50 concentration of *Carybdea alata* venom, the immunized animals were able to tolerate the venom of jellyfish with the above concentrations and remained alive for 30 days.

Discussion

In current study, the CfTX-1 recombinant protein showed protective activity against jellyfish venom. Raw venom of jellyfish is a combination of various proteins with an antigenic and irreversible specifies that can increase the production of polyclonal antibodies. Also these proteins cause death by affecting the heart cells (14). CfTX-1 is considered as a dominant immunogenic determinant and can be candidate for producing recombinant vaccine (12). The separation of CfTX-1 and CfTX-2 proteins is very difficult due to their high homology (15, 16). Since the isolation of these proteins is very difficult and production of non-homologous protein sequences is of particular importance. In recent years, the full sequence of CfTX-1 and CfTX-2 has been cloned, expressed and immunization levels have been measured in the mice by other researchers (6).

The compounds in the jellyfish venom stimulate the cellular and humoral immune system (12). The symptoms of bites effects of *C. fleckeri* venom on hamster and mouse are similar to those of bites in humans (17). Here, we injected the synthetic immunogenic protein into mouse in four periods. The antibody level was evaluated. The mouse were immunized 50x LD50 of jellyfish poison, although at high doses of venom injections, only neuromuscular symptoms such as temporary muscle contraction were seen in mouse. It takes at least 60 min which antidote neutralize the effect of venom. Since CFTX proteins cause death in cells particularly in the heart cells, the amount and also the timing use of the antidote are of great importance (12). In most cases, the venom of box jellyfish can cause death in less than 5 min with effect on the heart (17). Other studies have also shown the efficacy of CLS box jellyfish antivenom with antibodies against nematocyst-derived *C. fleckeri* venom (18-20), but what is clear is that for treatment or prevention, special attention should be paid to the rapid effect of venom and its cardiotoxic effects, which sometimes leads to death. Also the design of the vaccine to provide immunity to individuals who are more exposed to jellyfish bite could be a major proposition.

Therefore, in this research, synthetic protein could protect mousses by 50 times the LD50 of the venom. The CFTX synthetic protein can be considered as a vaccine for the safety of individuals. What is certain is that most studies have been conducted to treat the jellyfish bite to produce antidotes. However, the time and dose of injection are important in the use of antidotes, although in some cases the use of an antidote is associated with immediate and delayed allergic reactions (21). Therefore, one effective way to reduce the biting of the jellyfish is to use an effective vaccine to protect against it (22) and is one of the important issues of the production of CFTX protein, which is suggested as an option to design an effective vaccine. However, more research is needed, especially regarding the extent studies to which it is effective and time stability in the body.

Conclusion

Concerning the antigenicity of CFTX-1 synthetic protein and the production of antibodies in the animal, this protein can be used as a candidate for vaccine to get concurrent immunization against the Jellyfish venom.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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