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Original Article

Production and purification of polyclonal antibodies against Diphtheria toxin

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Abstract

Diphtheria is a fatal disease caused by exotoxin of *Corynebacterium diphtheria*. This toxin consists of two chains, catalytic chain (A) and binding (B) chain. By binding chain (B), the toxin binds to its receptor on numerous body cells such as myocardial, kidney and peripheral nerve cells. After entering, catalytic chain (A) inhibits protein synthesis and finally can cause cell death. At this time, the toxoid form of diphtheria toxin is used as vaccine. The aim of this study was the immunological analysis of the mutated synthetic catalytic subunit of diphtheria toxin in laboratory animals as a vaccine candidate, in addition to polyclonal antibody production and purification against diphtheria toxin. For this purpose the dtx recombinant protein (with two mutant: A158G and G52E) was expressed using pET28a/dtxA plasmid in *E. coli* Bl21DE3 host. Then, recombinant protein, as a candidate vaccine, was extracted and purified. After evaluating and confirming the protein by SDS-PAGE and western blotting, immunization carried out in laboratory animals. Finally, followed by antibody titration by ELISA, antibody purification performed as well. The mutated recombinant protein prepared from an optimized expression was extracted and purified. Then, this protein was confirmed by SDS-PAGE and western blotting. ELISA results showed a satisfactory immunization of animals by this protein. Polyclonal antibody production and purification against diphtheria toxin was performed by G protein column and confirmed by ELISA. ELISA results showed a high titer of polyclonal antibody against diphtheria toxin in animal's serum after immunization by recombinant DTx protein.

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Introduction

The most important species in Corynebacterium family is *Corynebacterium diphtheria*, the agent of diphtheria. It was found by Edwin Klebs in membrane over the tonsil of the patients with diphtheria in 1883 and its characteristics were described. In 1899, Williams revealed that *Corynebacterium diphtheria* PW8, type mitis, produces more and better toxin than the others, therefore, most studies have been done on this type [1-3].

Diphtheria is an acute respiratory disease with high mortality rates in young children and older people. The use of diphtheria toxoid during the past half century could inhibit this disease in the world. Pathogenic potential of the bacteria arises from the exotoxin effect, not by bacterial invasion. So after infection created in the upper respiratory tract, especially in the throat and sometimes in nose, larynx and trachea, meanwhile releasing the toxin produced by the bacteria in the body, cause damage to the tissues, especially the kidneys, myocardium, or peripheral nerves and kidneys. However, sometimes the obstruction of larynx and trachea, by the development of a false membrane, due to the enormous growth of bacteria can lead to severe breathing problems and endanger the patient's life [1-4].

The toxoid of this toxin is applied with 95% efficiency for active immunization. Although it is essential and effective to treatment the patients with diphtheria using an equine antiserum containing diphtheria antitoxin, usually the antibiotics can lead to eliminating the bacteria, though cannot be used in place of antitoxin [5-7].

It is not needed that bacteria enter to the bloodstream to create the disease symptoms, since the toxicity is directly depends on exotoxin secretion rate. *Dtx* gene, encoding Diphtheria toxin (DTX), can be entered into bacteria by lysogenic bacteriophage ß. The toxicity effect of heat-labile protein toxin with a molecular weight of approximately 61 kDa, can be reduced in light, chemical agents, and over time [8, 9]. Diphtheria toxin consists of two chain, linker chain (B) and catalytic chain (A), binding together by two disulfide bridges. The bond will be discrete in the presence of a reducing agent and protease enzymes or trypsin. The heat-labile linker chain, with a molecular weight of approximately 40 kD, causes the toxin to bind to the surface receptors on the target cells membrane. These receptors include an epidermal factor which binds to heparin, which is on many eukaryotic cells particularly neurons and heart cells, B chain usually has a hydrophobic translocate domain (called T domain) which can penetrate the membrane due to this property. The effect of diphtheria toxin is due to the catalytic chain, a heat-labile 192 amino acids protein with molecular weight of 21 kDa [10, 11]. There are two mechanisms of toxin

entry into host cells. In the first mechanism named direct entry, B subunit binds to the specific receptor on the host cell surface, creates a pore in the membrane through which the A subunit (DTxA) is transferred into the cell cytoplasm. The second mechanism is through receptor-dependent endocytosis, something like phagocytosis. The endocytosis induced by receptor bounded to the toxin leads to the toxin endocytosis and creates an endosome into the cell. The endosomes pH rapidly decreases by Hydrogen-Ion entrance into it which causes the A+B subunits to separate. Then subunit A passes through the endosome membrane and transferred into the cell cytoplasm, but B subunit remains in the endosome and is recycled to the cell surface [12-14].

Diphtheria toxin A subunit substrate is EF-2, a major factor in the protein synthesis elongation. Diphthamide is a modified histidine amino acid in EF-2 protein for the activity of A subunit. Active A subunit binds to NAD and forms ADP-ribose complex. The binding of complex to EF-2 brings protein synthesis to a halt and leading to cell death. Followed by protein synthesis cessation, DNA replication and RNA transcription are also blocked. Except Diphtheria toxin, there are many other toxins that block protein synthesis and lead to cell death, with the difference that Diphtheria toxin B subunit is able to bind to the receptor on the wide range of cells in the body, such as the eyes, liver, heart and kidney [15-17]. So far, Diphtheria toxin purified from *Corynebacterium diphtheria* PW8 culture medium and the application of formaldehyde for its conversion to toxoid are performed to produce recombinant vaccines and animal antiserums against diphtheria [19-21]. Researchers recently focus on recombinant production, which has abundant advantages as follows: the possibility of the production of recombinant subunit toxin, High production volume, the cost-effectiveness, the production under controlled commercial conditions, toxin production in high titers that provides the need for DT and DPT vaccines, the less problems for sterile conditions, using one equipment to produce a variety of vaccines, the reduction of experimental work and required space [22, 23]. One of the most important loci of point mutations studied is CRM197, where G52E amino acid substitution leads to changes in the spatial structure of active site, resulting in the loss of its toxicity and the lack of need for treatment with formaldehyde and also readily purification.

It also strongly decreases protein degradation and makes it resistant to the action of proteolytic enzymes. Glycine replacement by Alanine at position 158 is of major studied point mutations [10, 15, 22, 24-27].

In this study, for the first time, dtxA subunit gene is designed with two simultaneous point mutations G52E and A158G. It seems that these two mutations decrease the toxicity as close to zero as possible, while could stimulate the immune system and induce immunity as native toxin. This study is aimed to use a recombinant diphtheria toxin A subunit as a vaccine candidate and its use in animal antiserum production, and polyclonal antibodies purification against diphtheria in laboratory phase.

Materials and methods

Production and Purification of DTxA Protein

E. coli BL21(DE3) was used as a host cell. pET28a/dtxA plasmid was used to express recombinant DTxA Protein. After the initial expression of desired gene, the crude extracts of cells expressing protein were analyzed on SDS-PAGE gels. The expression of recombinant protein was studied in optimal conditions (induction under 1 mM isopropyl-D-thiogalactopyranoside (IPTG) when cells were grown to an $OD_{(600_{nm})}$ = 0.6, incubation at 37°C for 4 h). Recombinant protein was also investigated by Western blotting analysis. Sediment isolated from crude extract containing the insoluble protein was used for purification. The protein was purified using affinity chromatography and pH gradient buffer containing urea in Ni-NTA column (manufactured by kia Gen Co.). Effluents collected from the column were analyzed by 12% gel SDS-PAGE. The concentration of obtained proteins was determined by the Bradford method.

Purified protein confirmation by ELISA

ELISA technique was used to confirm the recombinant protein purified from Anti DTxA. Serial dilutions of purified protein, 5 µg to 156 ng, were loaded into the wells. 1:1000 dilution of Anti DTxA antibody and 1:2000 dilutions of rabbit antibody-conjugated were used.

Evaluation of immunogenicity

To evaluate the immunogenicity in a mouse model, 500 µl sterile PBS buffer was added to 50 mg purified protein followed by adding the same volume of complete Freund's adjuvant, the final volume was brought up to one milliliter. 5 mice were considered for injection and 200 µg/l of the mixture prepared above (containing 10 µg antigen) was injected subcutaneously into each mouse. A 5-member group was injected with sterile PBS buffer as control to verify the results. Subsequent injections were done as Table 1.

Table 1. Time, manner and protein concentration in immunization procedures

Dose	Day	The adjuvant type	the amount of antigen (µg/mouse)
1	1	Complete	10
2	20	incomplete	10
3	34	incomplete	10
4	48	Without adjuvant	10

To evaluate the immunogenicity, blood sampling was performed from the animals' eyes (immune and non-immune). Blood samples were incubated at 37°C for 1h followed by overnight storage at 4°C.

To separate the serum, the blood clot was removed, and then the serum was centrifuged at 1000 rpm at 4°C for 10 min. The clear yellowish fluid obtained was isolated for use in subsequent steps. Then the polyclonal antibodies present in the serum were analyzed by ELISA. The test was repeated three times.

Polyclonal antibodies purification

The purification of polyclonal antibodies was performed with recombinant DTxA antigen using G column. For this

purpose, the column was first washed three times with 5 ml of 100 mM Tris (pH:8) and 10 mM Tris (pH:8), the column effluent was collected. Then, 1M Tris solution was added to the 0.1 of serum volume followed by vortex and transferring to the column. Then the column was washed with 5 ml of 100mM Glycine solution (pH:3) three times and the column effluent was collected. Bradford method was used to determine the concentration of collected samples, which were finally analyzed on SDS-PAGE. After purifying the antibody and determining the concentration. A serial dilution from 2 mg/ml antibody was prepared and used for ELISA. To assess the detection of diphtheria toxin by antibodies purified, diphtheria toxoid in the vaccine retrieved from the serum of Razi Institute was used. Toxoid samples were used as antigens in ELISA.

Results

The expression of dtxA gene and the confirmation of protein expression

A sharp protein band about 25.4 kDa relating to the *Dtx A* was only observed in the induced cells, while no DTxA protein band was detected in non-induced cells (Fig. 1).

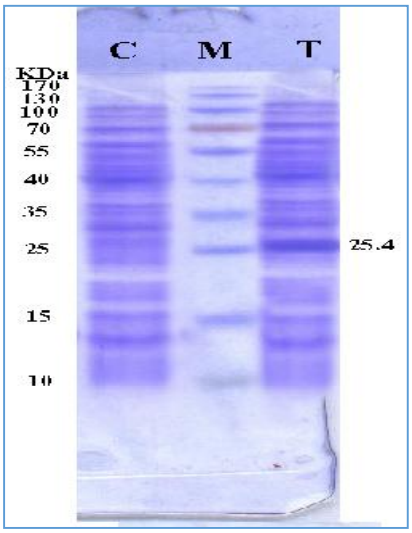

Figure 1. Investigation of DTxA protein expression on SDS-PAGE. Lane M: Protein marker SMO671, Lane C: Negative control (non-induced cells), Lane T: Induced cells with IPTG.

Denaturation method was applied to study the protein in soluble or inclusion body forms. After sonication and cell fractionation, the supernatant was isolated by centrifugation followed by treatment with B Buffer (containing urea) and passed through a nickel column. Gel electrophoresis showed a low amount of protein in the solution phase and a high amount of protein in the sample relating to the B buffer revealing the inclusion body forms. The recombinant protein with high purity was observed in the samples collected from final stage of washing and MES buffer (Fig. 2).

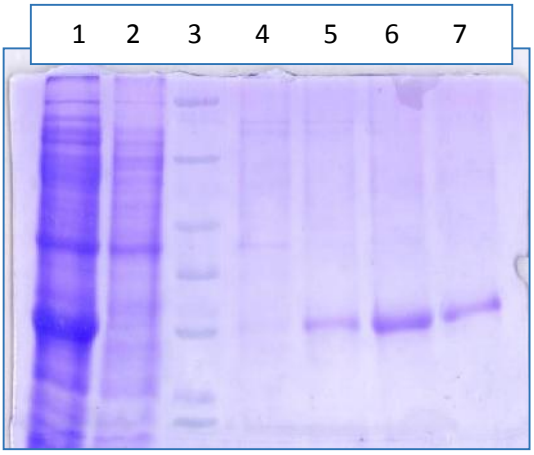

Figure 2. Evaluation of DTxA protein solubility. Lane 1: Crude extract of induced cells; Lane 2: Sample passing through the column (Flow); Lane 3: sm0431 Marker; Lane 4: Effluent relating to C buffer; Lane 5: Effluent relating to D buffer; Lane 6: Effluent relating to E buffer; Lane 7: Effluent relating to MES buffer.

Confirmation of recombinant proteins using immunoblotting technique

DTxA protein expressed in pET vector contains six histidine amino acid tags, therefore, Western blot technique using Anti-His tag antibodies was applied to confirm the recombinant protein. Figure 3 shows that the antibodies could identify the protein encoded by dtxA gene.

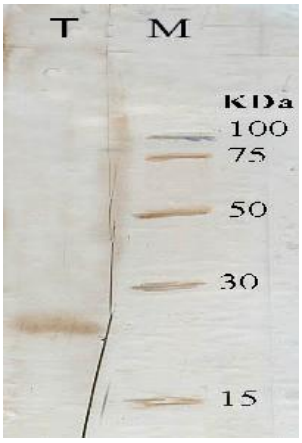

Figure 3. Confirmation of DTxA protein using immuno-blot technique: Lane T: Test samples of ELISA and Control by 1:5000 dilution of anti-histidine; Lane M: Protein Marker

Confirmation of the purified protein using ELISA technique

ELISA technique by using Anti DTxA was applied to confirm the purified protein. The results showed the antibody reaction with very low levels of the protein and the accuracy of desired protein (Fig. 4)

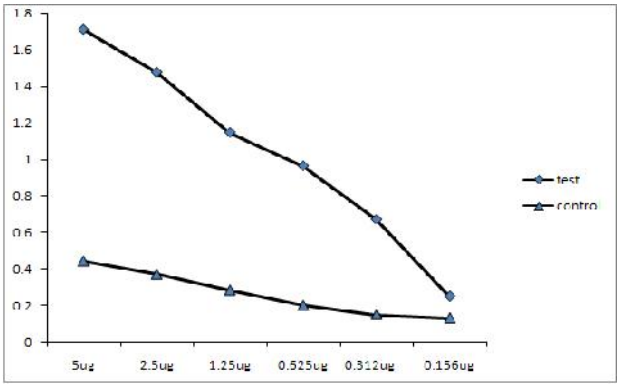

Figure 4: Confirmation of purified protein using Anti DTxA in ELISA test (Y: Absorbance; X: Protein Concentration)

ELISA results for antibodies against DtxA

Immunization was performed by DTxA injection into 5 mice. At intervals of injections and two weeks after the last injection, the mice were bled, and ELISA was done by prepared serum. The last results obtained followed by three times repetition is presented. Figure 5 shows ELISA relating to the DTxA purified protein (5 µg per well) and mice's serum. The first diagram shows the serum from the first bleeding (one week after the second injection). In the second and third diagram, the serum from the second bleeding (one week after the third injection) and the serum from third bleeding (one week after the fourth injection) are shown, respectively, with serial dilutions from 1:500 to 1:16000. As indicated in the diagram, after each injection, the levels of antibody produced in mice was increased, so that the absorbance was shown higher than 3/5 in ELISA from the third bleeding in dilutions lower than 1:500.

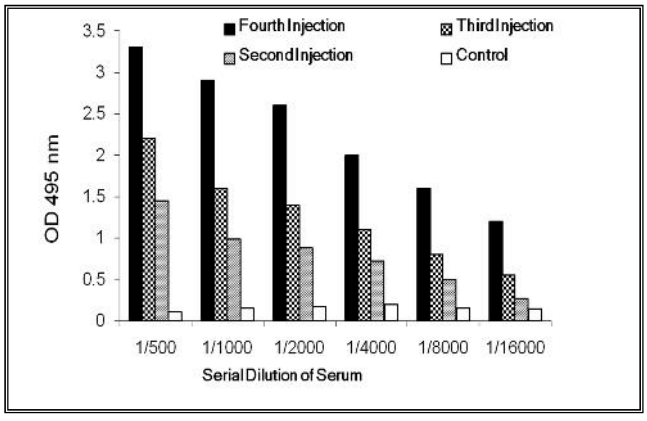

Figure 5: ELISA test results of antibodies derived from immunization. Evaluation of the antibody titer produced in mice against recombinant DTxA.

Purification of polyclonal antibodies

IgG antibodies, from the immunized mice's sera, was purified using column G and the results were confirmed by gel electrophoresis. The concentration of purified antibody was calculated 2µg/µl according to Bradford method.

Discussion

Roux and his colleagues reported that the main cause of diphtheria is its potent toxin, in 1888, therefore, its toxoid can be why it can be encapsulated using an effective treatment for the disease [3]. Behring also could obtain the diphtheria anti-toxin from the blood of guinea pigs immunized with toxoid. Active immunization against diphtheria was also conducted by Smith and colleagues in 1907, and the toxoid of diphtheria toxin was reported as vaccine by Ramon [4]. So far, various methods and media are conducted to produce the diphtheria toxin. In 2001, Sandaran and his colleagues studied the production of diphtheria toxin in a fermenter (with a simple design) [11]. Overloaded iron in broth, which prevents the release of toxins, can be removed using Baker's yeast [8, 9, 26, 27]. Taghavi Moghadam and his colleagues, in 2008, used the traditional processes of fermentation to produce the diphtheria toxin and to convert into toxoid. The significant increase in the production rate can provide the country requirements in DT and DPT vaccines production [22, 23, 28, 29]. Diphtheria toxoid vaccines are still used against the diphtheria in our country and many others developing countries. However, with the advent of the advanced techniques of biotechnology and genetic engineering, the interest of scientists has been increasing to apply this technology. These vaccines have undeniable superiority compared to second generation vaccine (recombinant) and third generation or even fourth generation. Therefore, it seems that the use of first-generation vaccine is facing extinction. This process has led to the licensing of new recombinant vaccines in some European countries. Several studies in France, America and Italy during 1997 to 2010 on the design, fabrication and investigation of different recombinant proteins as vaccine candidate against diphtheria, and even the use of nanobiotechnology in the production of oral or breathing vaccine, with more efficiency than first-generation vaccines, are only a small part of studies being conducted in the world [15, 22, 23, 24, 30, 31, 32]. Hence, it is essential that the researches in this field to be followed in our country. Therefore, this study is aimed to design the recombinant protein containing the only a small part of the native protein, which has following feature:

I. To stimulate the immune system against natural toxins and develop a complete immune response by appropriate antigenic properties of the protein can.

II. To choose this part as a smaller subunit of the toxin to stimulate the immune system and induce immunity similar to toxoid.

III. To reduce the toxicity by the single point mutation in the active site, and change in the spatial structure of proteins.

VI. To facilitate the manipulation due to shortening the gene sequences.

Conclusion

In this study, the *E. coli* Bl21 DE3 was transformed by pET28a/dtxA followed by protein expression and purification and confirming test. After injection to laboratory animals, the titer of antibodies isolated from the animal's blood was performed using ELISA. Consequently, the amount of antibody produced in mice was increased after each injection, and finally 2 µg/µl antibody was purified by G column.

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