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Characterization of antibody titer and immunogenic feature of light chain of botulinum neurotoxin type A

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ABSTRACT

The botulinum neurotoxins (BoNTs), the most toxic biological compounds, are known to cause muscle paralysis. These toxins are enzymatic properties which inhibit the release of the acetylcholine. The purposes of this study are the recombinant expression of light chain (LC) of botulinum toxin types A (rBoNT/A-LC) with high purity, the evaluation of its antibody titer yield and its immunogenicity, and finally the evaluation of in vitro reorganization of BoNT/A-holotoxin by produced antibody. BoNT/A light chain gene sequences were obtained from the NCBI genome database. After codon optimization of target gene to better express in E. coli, the target gene was ordered to be synthesized in pET-28a (+). E. coli BL21 (DE3) was transformed by the mentioned vector containing the gene. The expression process was performed in standard conditions. In order to express the recombinant protein in soluble form, optimization of host cell culture and expression process was performed. The protein was purified by affinity chromatography (Ni-NTA column) and was confirmed with specific antibodies. In this study, the highest expression of soluble protein was obtained at 0.5 mM IPTG, cell culture optical density of 0.5, and the induction time of 18 h at 18 °C. Western blot analysis confirmed the target protein. The results showed that rBoNT/A-LC was highly expressed in soluble form and highly purified using affinity chromatography. Immunology results showed that the immune mice with rBoNT/A-LC are able to endurance up to 100 LD_{50} of BoNT/A. The results also indicated that the produced antibodies can recognize up to 200 ng of BoNT/A-holotoxin.

Keywords: Botulinum neurotoxins type A (BoNT/A), Light chain (LC), Recombinant protein

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INTRODUCTION

Clostridium botulinum, a gram-positive anaerobic bacterium, is found in soil [1]. Among the seven known serotypes of neurotoxins that include A, B, C1, D, E, F, and G, only types A, B, E, and rarely F are involved in human botulism [2]. The most durable form of paralysis is caused by botulinum neurotoxins type A (BoNT/A) [3]. The Lethal dose (LD_{50}) of BoNT/A for a 70 kg-human through inhalation and injection, is estimated to be 70 µg and 0.9 µg, respectively [4]. Each of the botulinum neurotoxins is produced as a single chain protein with a weight of 150 kDa and then divided to two chains; a 100 kDa-heavy chain (HC) and a 50-kDa light chain (LC), which remains connected to each other by a disulfide bond. The light chain is responsible for binding to peripheral cholinergic neurons and LC is a zinc-dependent endo-peptidase which specifically digests involved proteins in neurotransmitters release [5, 6, 7]. Studies suggest a four-step mechanism of cell poisoning by botulinum neurotoxins as follows: Toxin binding to the membrane of motor neurons, toxin penetration into the membrane of motor neurons, toxin passing from neurons membrane with disulfide bond reduction between two chains, and intracellular catalytic activity by light chain [6]. Botulinum neurotoxins have three types of substrate; Synaptobrvin or VAMP (Vesicle Associated Membrane) which is the substrate of serotype F, D, G, and B, SNAP25 (synaptosomal associated protein of 25 kDa) which is the substrate of serotypes A, C, and E, and finally Synataxin

which is affected by serotype C [8]. The reports show that BoNT/A affect SNAP - 25 and digest it on Gln^{197}-Arg^{198} [9]. From structural point of view, BoNTs light chains are globular proteins with 36.5% sequence similarity [1]. Most metalo-peptidases need zinc (Zn^{+2}) as an essential metal ion for their catalytic activity and contains three amino acids and a H_2O molecule in their active site, which are attached to Zn^{+2}. The first two ligands of BoNT/A active site are histidines, which are located within the HEXXH motif and are needed for to stabilize H_2O molecule. The glutamic acid of HEXXH motif is responsible for the BoNT/A catalytic activity. Biological activity and the tertiary structure of BoNT were demolished Zn^{+2} removal, so Zn^{+2} plays an important role in maintenance of the biologically active structure of BoNT/A [10]. Basis for botulism treatment are health care and passive immunization by equine anti-toxins. Early administration of these anti-toxins prevents or reduces severity of nerve damages, but it is unable to restore the already paralysis [11]. Currently, vaccination against this bacterium is performed using formaldehyde-inactivated toxins [12]. For this purpose BoNT/A and E holotoxines are produced and inactivated by formaldehyde, then the resulting toxoid vaccine was used [13]. However, toxoid vaccination has several drawbacks that vindicate the attempt to develop newer vaccines. In newer vaccines, genetic-engineering techniques are applied and certain areas of the toxins that stimulate the immune system and evoke a large amount of antibodies are targeted. Here we focused on the light chain of BoNT/A and expressed the recombinant form of this chain, and evaluate the antibody titer and LC immunogenicity in mice. Then the rate of BoNT/A holotoxin reorganization by anti-rBoNT/A-LC antibodies were evaluated.

MATERIALS AND METHODS

Synthetic gene of rBoNT/A-LC on pET-28a was prepared from shinegene (China). Restriction enzymes and IPTG were obtained from Fermntaz (Ukraine). *E. coli* BL21 (DE3) bacteria and pET-28a were obtained from Novagen (Iran). The materials which were used in SDS-PAGE and Western bloting, as well as Ni-NTA column were purchased from Qiagen (Iran). Swinx Millipore filters (cut off number 50 kDa) were used.

Transformation of *E. coli* cells by the recombinant plasmid pET28a-LC

First, after *E. coli* cells culturing in LB medium, competent cells were prepared by chemical methods. The, recombinant plasmids were transferred into competent cells by cold shock procedure. After overnight culturing on Mack-Koncky agar medium containing kanamycin, the transformed cells were screened. Among the colonies that were able to grow in the medium with kanamycin a few colonies were grown in LB medium randomly. Then their plasmid was purified [14]. Considering the restriction enzyme *Nde*I and *Xho*I, taking place at the beginning and end of the gene in the vector, to confirm the presence of the gene in purified plasmids, dual-enzyme digestion was performed. The enzymatic digestion was characterized on 1% agarose gel.

rBoNT/A-LC expression in *E. coli* BL21-DE3 cells

First, the protein expression was performed in standard conditions. Since rBoNT/A-LC expression in standard condition resulted in producing inclusion bodies, different conditions were evaluated to optimize the expression process. After applying each condition, the cells were centrifuged (5 min, 5000 rpm). The collected cells were suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, 1 mM PMSF, pH = 8.0) and followed by sonication (75% power, 4 cycle: 10 sec with 15 sec intervals in ice). Cell lysates were centrifuged at 14000 rpm for 15 min. The supernatant was removed and the pellet was resolved with an equal amount of lysis buffer. The concentration of all samples was estimated by Bradford method using BSA as standard protein. Both supernatant and pellet were electrophoresed on 12% SDS-PAGE and visualized with Cumassi blue [15].

Purification of rBoNT/A-LC

The recombinant protein purification was performed using Ni-NTA affinity chromatography. 1500 µl of each sample was added to the Ni-NTA column. After the column preparation, different concentrations of Imidazole (20, 40, 120, 170, 250 mM) were added to the column and collected fractions were studied by 12% SDS-PAGE [16].

Filtration

In order to more purify of rBoNT/A-LC, filtration was used. For this purpose 1500 µl of each sample was added to a falcon containing a Millipore filter with cut off number 50 kDa and centrifuged at 4 ºC, 3 h, and 2000 rpm.

Recombinant protein confirmation by ELISA and Western blotting

In order to verify rBoNT/A-LC, Western blotting was used. For this purpose 10, 20, and 30 µg of the protein were run on SDS-PAGE and then transferred to Nitrocellulose paper and analyzed using polyclonal equine anti-BoNT/A IgGs. The protein confirmation was also analyzed by ELISA.

The recombinant protein preparation and its injection into mice

In order to produce antibodies against rBoNT/A-LC, five mice as test and two as controls were used. In the first injection, 30 µg of the purified protein was solved in sterile PBS up to final volume of 400 µl and combined with equal volume of complete Freund adjuvant. 100 µl of resulted solution was subcutaneously injected into each mouse [16]. On days 14 and 21 the same amount of protein was combined with incomplete Freund adjuvant and subcutaneously injected into mice. A week after the last injection the mice were bled. Blood samples were incubated at 37 ° C for 30 min. Blood serum was obtained by centrifugation for 10 min, 3000 rpm.

Table 1 - Time and conditions of the recombinant protein injections

No. of injection	Day	Amount of injected antigen (µg)	Type of Freund adjuvant	Type of injection
1	1	30	Complete	Subcutaneously
2	14	30	Incomplete	Subcutaneously
3	21	30	Incomplete	Subcutaneously

Measurement of produced antibody titer using ELISA technique

The anti-rBoNT/A-LC IgGs titer was determined by indirect ELISA technique. 20 µg/µl of the recombinant protein in carbonate buffer (0.05 M pH = 9.6) was prepared and divided to plates wells (each 100 µl). The plates were incubated overnight at 4 °C and then were washed four times with PBST and dried (washing and drying the plates at the end of each stage was done). 3% gelatin was used to block wells and plates were incubated for 1 h at 37 °C. Serial dilutions of the mice sera started with 1:500 dilution was added to the plate wells and incubated at 37 ° C, 30 min in room temperature. Then 1:2000 dilution of the mouse conjugated was added to each well and the plates incubated for 30 min at 37 ° C in room temperature. The substrate solution (containing citrate-phosphate buffer, OPD, H_2O_2) was then added to each well and finally the reaction was terminated by adding 2.5 N H_2SO_4 and the plate was read using ELISA Reader at 495 nm. In order to detect the BoNT/A toxin by produced antibodies, 2 µg of the protein was added to each well and assayed by indirect ELISA according to the above mentioned procedure.

RESULTS

Transformation of *E. coli* BL21-DE3 cells

After transformation of *E. coli* cells with pET-28a containing the synthetic gene, in order to confirm the presence of the gene, the plasmid was purified and digested with *Nde*I and *Xho*I enzyme, and a 1278-bp fragment was obtained on agarose gel indicating the presence of the gene.

The recombinant protein expression and its optimization

After ensuring that the desired gene is presented in the plasmid, the expression was performed using 1 mM IPTG and induced for 5 hours at 37 °C. The supernatant and pellet of centrifuged cells were electrophoresed on 12%SDS-PAGE and stained by coomassie blue.

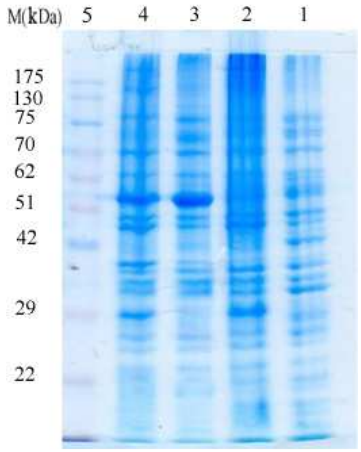

Figure 1 - Evaluation of the expression of test and control samples at optimum conditions. Column 1: The control supernatant without induction with IPTG. Column 2: The control sediment, without induction with IPTG. Column 3: The test supernatant, induced with IPTG. Column 4: The test sediment, induced with IPTG. Column 5: protein molecular weight marker.

The expression result, which is shown in figure 2, indicated that rBoNT/A-LC is expressed as inclusion bodies. Therefore, in order to produce a soluble form of protein, the expression process in different conditions (according to

Table 1) was evaluated. Eventually, the expression condition of 0.5 mM IPTG, 18 h incubation at 18 °C [5] with 20 µM $ZnCl_2$ led to a soluble form of the protein (Figure 1).

The recombinant protein purification

After expressing of the soluble proteins, purification was performed using affinity chromatography at 4 °C. The results are displayed in Figure 2.

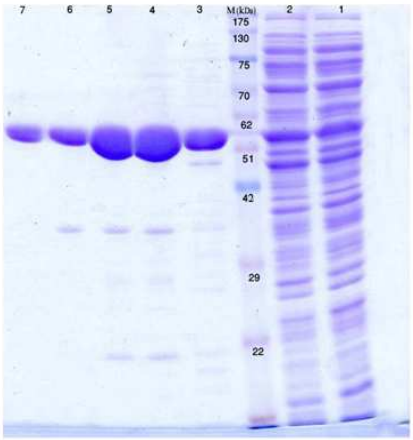

Figure 2 - Purification of proteins using affinity chromatography and filtration. Column 1: 10 mM Imidazole, which has passed through the column. Column 2: 20 mM Imidazole, which has passed through the column. Column M: protein molecular weight marker. Column 3: 40 mM Imidazole, which has passed through the column. Column 4: 120 mM Imidazole, which has passed through the column. Column 5: 170 mM Imidazole, which has passed through the column. Column 6: 250 mM Imidazole, which has passed through the column. Column 7: the solution passed through the filter.

Confirmation of the expressed protein

The purified protein was confirmed using Western blot. The results are displayed in Figure 3.

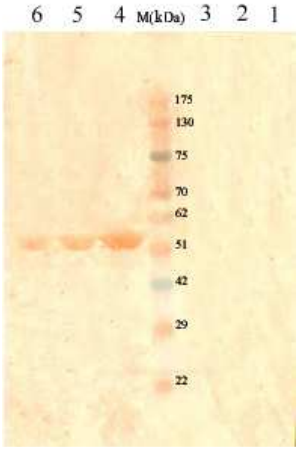

Figure 3. The confirmation of rBoNT/A-LC by Western blotting. 10, 20, and 30 µg of BSA was loaded as negative controls, in columns 1, 2, and 3, respectively. Column M (kDa): protein molecular weight marker. 10, 20, and 30 µg of the purified protein was loaded in column 4, 5, and 6, respectively.

Antibody production assay using ELISA technique

After 10 days of each injection, the mice were bled and the sera were separated antibody produced titer in each phase was determined using ELISA technique (Figure 4).

Mice challenge with active BoNT/A

The minimum lethal dose of toxin (LD_{50}) was determined before the experiments. In this study, two groups of mice (each with five mice) were used. A group which was injected with 1 and 10 LD_{50} of BoNT/A were all survived. Three of 5 mice which were injected with 100 LD50 of BoNT/A, died after 48 h. All mice died after injection of 1000 LD_{50}. All control mice which received 1, 10, 100, and 1000 LD_{50} of BoNT/A died.

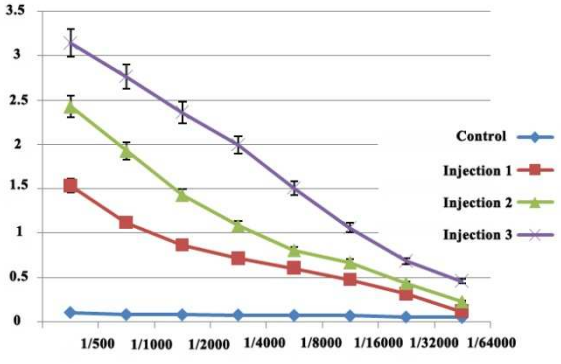

Figure 4 - The mean antibody titer after each injection to mice

BoNT/A holotoxin identified using the produced antibodies *in vitro*

In order to detect the BoNT/A holotoxin with the produced antibodies, 2 µg of BoNT/A was added to the first well and then, serial dilutions were prepared same as above mentioned method. Indirect ELISA were used to assay the results (Figure 5)

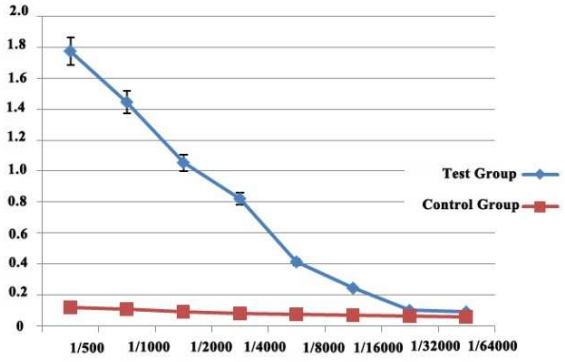

Figure 5 - ELISA experiment for detecting of BoNT/A holotoxin by the produced antibodies

DISCUSSION

Three types of BoNTs which cause botulism in humans are more important for researchers. The most researches on Botulism, have focused on the field of endopeptidase effect of BoNT/A, B, and E, which cause human botulism [17]. In this study we tried to produce a soluble protein with high purity that can be used in our next studies. For this purpose, the nucleotide sequence of BoNT/A-LC of *C. botulinum* type A strain (ATCC 3505) were obtained from Gene Bank. According to the study of Baldwin [18] that had used the 425 aa-sequences (due to higher solubility and more stability of it in comparison with 448 aa- sequence protein) used, we also chose the nucleotide sequence of 425 aa- sequence protein. Since *C. botulinum* genes are weakly expressed in *E. coli* [19-21], the rBoNT/A-LC nucleotide sequence was optimized based on the common codons in *E. coli*. Due to performed optimization in this study, the GC% in the desired sequence increased from 28 % to 46 % and also the rare codons were deleted. Considering that the GC% of botulinum genes is about 30 % on average, therefore the optimization significantly has increased the recombinant protein expression in the host cells [22]. Finally, the gene was inserted into pET28a expression vector with the His6-tag. The presence of this tag assists to confirm and easy purify of the expressed protein. The recombinant protein was highly expressed. However this high expression caused some problems such as not enough time for proteins to fold correctly which leads to creation of inclusion bodies that should be altered to soluble form. It was tried to remove or lower the production rate of inclusion bodies. Eventually soluble form of the protein was obtained at 18 °C, 0.5 mM IPTG [5] and in the presence of 20 µM $ZnCl_2$. In addition, zinc, which plays an important role in the permanence of the biologically active structure of BoNT/A, was added to the culture medium of host cells [10]. Also at lower temperatures (below the optimum temperature of growth and expression) the growth kinetics and expression levels were decreased. Therefore a long induction time (18 hours) was necessary to obtain high level of expression. In this condition the host cells have enough time to correctly fold the recombinant protein and prevent the formation of inclusion bodies. Figure 1 shows the production of soluble protein and inclusion bodies that nearly 65 % of the protein was estimated to be soluble. When researchers try to purify botulinum neurotoxins, they may be encountered with different problems such as protein exit from the chromatography column at low Imidazole concentrations. The researchers believe that it can be due to the His-tag burying in the recombinant protein and to

solve the problem, they suggest using the His-tag in the both side (N- and C- terminal) of the proteins [23]. Singh et.al, were confronted to this problem in BoNT- light chain purification, where an amount of the protein was exited from the column at 20 mM Imidazole, and also at concentrations above 100 mM imidazole, in addition to light chain, other proteins were also observed on SDS-PAGE. In order to better purification of the output of 100 mM Imidazole, these researchers passed the output through DEAE-A50 ion exchange column and thus they were able to remove impurities. But in the present purification the problem was not very acute, and the few remaining impurities were removed by filtration [24]. The protein solution at 250 mM Imidazole which includes an additional 38 kDa-band was passed through a filter with cut off number 50 kDa, thus we could remove the extra 38 kDa-band and improve the purity of the target protein. Then the protein was confirmed using Western blotting. In a research which was conducted by - Jensen et.al, the catalytic domain of the BoNT/A, both separately and fused with the translocation domain, produced *in vivo* and their immunogenicity was evaluated. The immunized mice with 5 and 15 µg of the purified proteins were able to tolerate 100 and 1000 LD_{50} of the toxin. It is notable that the separate catalytic domain did not have any significant immunogenicity difference with the fused protein. These researchers believed that recombinant catalytic domain is able to cause an acceptable immunity. However, they suggest using albumin as an appropriate adjuvant for immunization [25]. The lower immunogenicity of the present recombinant protein compared to the results of Jensen et.al. may be due to the type of buffer and adjuvant which is used. Our results showed that rBoNT/A-LC cause an acceptable immunogenicity in mice, and also indicated that produced anti-rBoNT/A-LC antibodies are able to detect 200 ng of BoNT/A holotoxin *in vitro*.

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