Original Article

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Evaluation of the Expression of Recombinant Type A Botulinum Neurotoxin Light Chain Loaded onto a Cell-Penetrating Peptide

P. Saffarian (MSc)[1], Sh. Najar Peeraye (PhD)[1], J. Amani (PhD)[2], A.A. Imani Fooladi (PhD)*[2]

1.Department of Medical Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, I.R.Iran
2.Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, I.R.Iran

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ABSTRACT

BACKGROUND AND OBJECTIVE: Botulinum toxin type A (BoNT/A) is widely used in the treatment of some muscle contraction disorders through injection. This study aimed to produce recombinant protein through covalent bonding of the light chain of BoNT/A to the peptide TAT (47-57), and to create an optimized condition for expression and purification of the protein.

METHODS: In this preliminary study, the nucleotide sequences of the light chain of BoNT/A and TAT peptide were obtained from Gen Bank, and genetic structures containing these sequences were designed and engineered. After cloning into the BL21 (DE3) E. coli vector, expression was induced by IPTG. Thereafter, optimum thermal condition and IPTG concentration for maximum expression were determined based on the difference in the intensity of staining between the bands on SDS-PAGE protein gel. The recombinant protein was purified through nickel column chromatography (Ni-NTA).

FINDINGS: The produced chimeric protein is insoluble in normal conditions (1 mM IPTG and at 37˚ C). Through optimization of expression conditions (0.5 mM IPTG and at 18˚ C) 60% of the chimeric protein was produced as solution.

CONCLUSION: Based on our results, through expressing the recombinant protein as a solution, the protein maintained its proper folding and function. Hence, the use of denaturation compounds for solution making and destabilization of folded protein structures is not required.

KEY WORDS: *Light chain of botulinum toxin type A, Cell-penetrating peptide, Recombinant protein*.

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***Corresponding Author: AA. Imani Fooladi (PhD)**
Address: Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, I.R.Iran
Tel: +98 21 88068924
E-mail: imanifouladi.a@gmail.com

Introduction

Botulinum toxin type A (BoNT/A) (150 kDa) is comprised of a dichain structure of light and heavy (1-3). Light chain (the second catalytic; 50 kDa), is a zinc-related metalloprotease, and the natural substrate of this enzyme-protein is synaptosomal-associated protein (SNAP-25; 25 kDa), which is responsible for transporting the vesicles containing excitatory neurotransmitters (4,5). Toxin inhibits the release of acetylcholine and temporarily prevents muscular contraction. In the recent years, botulinum toxin serotypes A and B were successfully used as biological drugs for reduction of facial wrinkles and treatment of some disorders implicated by impaired muscular contractile performance such as blepharospasm and migraines (6-11).

Despite the effectiveness of BoNT/A in the treatment of muscular defects, its injection leads to erythema, inflammation, irritation, pain, and sometimes bleeding in the injection area. Thus, applying non-invasive methods for safe prescription of this agent seems to be necessary. Cell penetrating peptides (CPP) have been widely used for direct transfer of molecules to cells (12, 13). The length of these peptides is generally less than 30 amino acids, and they are capable of introducing biological compounds (DNA, RNA, peptides, and proteins) into living cells in the form of covalent or non-covalent bonds (14). In 1991, it was found that Drosophila Antennapedia homeodomain is drawn in by neurons. This was the origin of the discovery of the first CPP, called pentrapin, in 1994 (15).

In the recent years, PsorBan®, a cyclosporine-poly-arginine conjugate, was developed as a topical treatment for psoriasis, and KAI-9803 conjugate was produced from C-TAT kinase protein for the treatment of acute myocardial infarction (16); TAT peptide (amino acids 47-57) is a CPP (15). This study aimed to produce a recombinant protein derived from BoNT/A through covalent coupling of BoNT/A with TAT peptide (47-57), and to provide an optimized condition for the expression and purification of this protein.

Methods

Gene preparation: Nucleic acid sequence of light chain of BoNT/A (nucleotides 1-1344) was extracted from the standard ATCC 3502 strain and TAT peptide (nucleotides 139-169), which belonged to the human immunodeficiency virus type 1, obtained from GenBank. The genetic construct of recombinant protein was designed and engineered. A poly-histidine (6xHis) was tagged to the 3' end of this sequence to help identify and purify the proteins. In addition, the HindIII and BamH1 sites of restriction enzymes was determined at the 5' and 3' ends of the recombinant sequence, respectively, so that they could be used when the need for extraction of the recombinant sequence from plasmid arose.

Gene cloning: The gene was digested with HindIII and BamH1 (Fermentas, Vilnius, Lithuania) enzymes. Gene fragment containing the desired sequence of the recombinant protein was combined with linearized plasmid pET28a using T4DNA ligase enzyme. Then, it was transferred to E. coli BL21 (DE3) as an expression host with heat shock (30 minutes in ice, 90 seconds at 42˚ C, and 1 minute in ice).

The bacteria were cultured on LB agar containing 20 µg/ml kanamycin, and were incubated for 24 hours at 37˚C. To confirm the insert into the plasmid, colony polymerase chain reaction (PCR) was performed randomly on the colonies grown on the agar using T7 promoter and T7 terminator of pET28a plasmid (CinnaGen Co., Tehran, Iran). The normal distance between the two primers is 300 bp; upon inserting the gene to the plasmid, 1446 bp is added to the PCR products (in total 1746 bp).

Plasmids of the colonies containing the insert were extracted using Plasmid Purification Kit (iNtRON, Korea), and then were digested with HindIII and BamH1 enzymes to confirm the insert into the plasmid. The selected colonies were kept at -80 ˚C for later use.

Expression of recombinant proteins: Colonies with the insert were cultivated for 24 hours at 37˚ C on 50 ml LB medium containing 20 µg/ml kanamycin. After $OD600_{nm}$ of the medium reached 0.6-0.9, protein construction was induced by adding 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). Then, the produced proteins were incubated at 37˚C for 24 hours in a shaking incubator at 150 rpm.

Afterwards, the cells were separated from the culture medium through centrifugation (5000 rpm for five minutes). Cell sediment was lysed using 8M urea lysis buffer, after sonication (30 cycles with rest intervals of 45 seconds on ice), it was centrifuged (14000 rpm for 20 minutes).

The supernatant containing soluble proteins and insoluble proteins from the lysate of each clone were isolated separately on SDS-PAGE gel. The thickness

of the protein bands was measured using Quantity One Analysis Software Version 4.6.5 (Bio-Rad, USA). The bacterial clones capable of protein expression were preserved at -80˚ C for later use.

Optimizing the expression: To optimize protein expression, the bacterial culture medium containing the insert was induced at different concentrations of IPTG (1 mm, 0.8 mm, 0.5 mm, and 0.3 mm). Afterwards, the induced bacterial culture was incubated at 37˚C and 30˚C. The induced culture media were evaluated in terms of protein production through 12% SDS-PAGE gel. In this study, we tried to increase protein solubility through changing and optimizing expression conditions. Therefore, protein expression was induced at low concentrations of IPTG (0.5 mm) and then was incubated at temperatures below 18˚C (17). Moreover, 0.25 µm $ZnCl_2$ was added to the bacterial culture as the mineral element to maintain the three-dimensional structure of the proteins.

Protein Purification: Debris of one liter of induced bacterial culture (0.5 mM IPTG at 18˚C) was incubated after 22 hours. Cell sediment was centrifuged after sonication procedure. To purify the proteins, the supernatant containing protein solution was collected and transferred to chromatography column containing particles of nickel (Ni-NTA agarose, Qiagen, USA) (17). The column was eluted with a solution containing 20 mM (Tris-HCl 20 mM and NaCl 500 mM) and 100mM imidazole. To purify the recombinant protein from the column, we used an elution buffer containing 250 mM imidazole. The flow of column were collected separately and electrophoresed on 12% SDS-PAGE gel.

Results

Gene cloning: The PCR products of each clone was seperated on the agarose gel. Clones 2,3,4, and 10 manifested a 1746 bp fragment which indicats the presens of inserted recombinant sequence (fig 1).

Recombinant protein expression: Among the four bacterial clones carrying the recombinant plasmid, the clone number four demonstrated appropriate expression of the protein after induction. SDS-PAGE gel image obtained from electrophoresis of the samples (fig 2) revealed that the recombinant protein was produced as an insoluble (inclusion), and that there was a very small amount of protein in the solution phase.

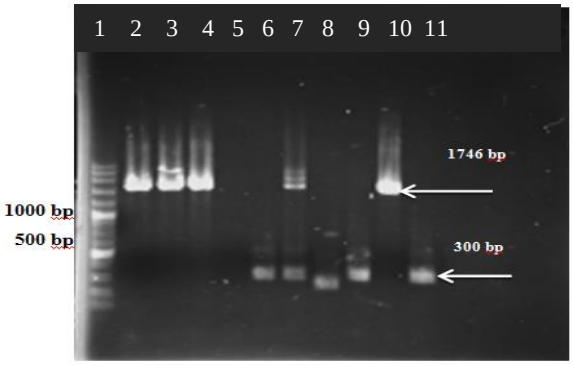

Figure 1. Electrophoresis of polymerase chain reaction (PCR) product of E. *coli* BL2 (DE3); among the randomly selected clones (columns 2-11), clones number 2, 3, 4, and 10 contained the transformed fragment. PCR was performed using T7 promoter and T7 terminator primers of pET28a plasmid. Column 1: DNA size marker.

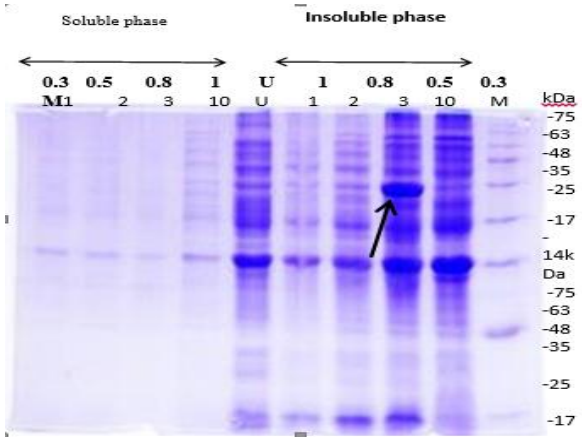

Figure2. Evaluation of protein expression in E. *coli* Bl21 (DE3); only clone number 3 (shown with an arrow) was capable of effective expression of the recombinant protein (54 kDa band), Solution phase: solution proteins, Insoluble phase: containing insoluble proteins, U: cell lysis before induction of expression, M: protein size indicator; 12% SDS-PAGE gel stained with Coomassie Blue G-250

Expression optimization: Upon optimizing protein expression, it was found that recombinant proteins yielded the highest expression at 1 mM IPTG concentrations and 37˚C (fig 3a and b). In order to optimize the conditions for the production of recombinant proteins in solution phase, we started to induce protein expression at 0.5 mM IPTG concentration and to incubate the bacteria at 18˚ C. A large amount of proteins (about 60%) was transferred to the solution phase (fig 4).

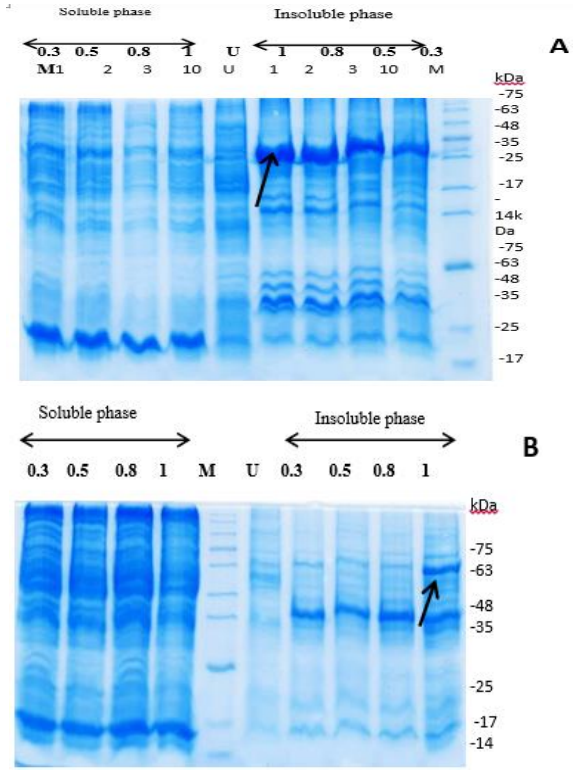

Figure3. Optimizing expression of recombinant type A botulinum neurotoxin light chain

The recombinant protein (band 54 kDA) at 1 mM IPTG concentration and 37˚ C had the highest expression rate. Solution phase: solution proteins, Insoluble phase: insoluble proteins, U: before induction of expression, M: protein size indicator; the band indicating the highest rate of expression is shown by the arrow. 12% SDS-PAGE gel stained with Coomassie Blue G-250

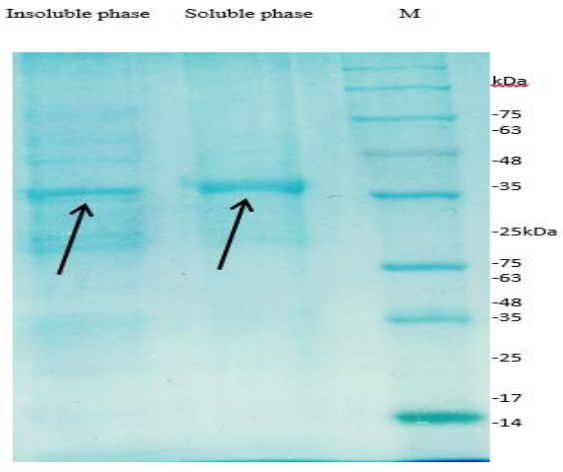

Figure4. The expression of recombinant type A botulinum neurotoxin light chain in solution phase.

Protein expression was induced by 0.5 mM IPTG. After incubation of the culture at 18˚ C, about 60% of the recombinant protein was produced in solution form, Solution phase: solution proteins, Insoluble phase: insoluble proteins, M: protein size indicator; the band indicating the highest rate of expression is shown by the arrow. 12% SDS-PAGE gel stained with Coomassie Blue G-250

Protein Purification: Each of the fractions obtained from protein purification were separately electrophoresed on SDS-PAGE gel in columns containing the resin-bound nickel. The rate of protein purification in the elution solution containing 250 mM imidazole was about 95% (fig 5).

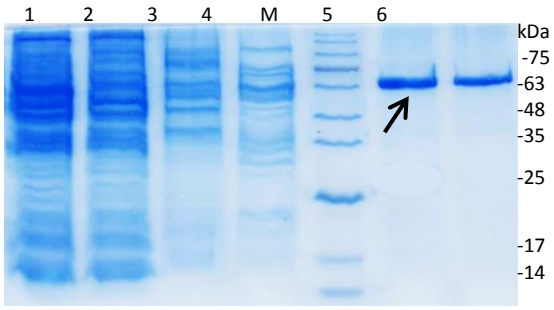

Figure5. Purification of the recombinant protein using nickel column chromatography; Column 1: before purification, Column 2: the initial current of the column, Columns 3&4: elution with a solution containing 20 mM and 100 mM imidazole, Columns 5 &6:purified proteins in a solution containing 250 mM imidazole; the band related to the purified protein is shown by the arrow. 12% SDS-PAGE gel stained with Coomassie Blue G-250

Discussion

In this study, the light chain of BoNT/A was covalently linked to TAT peptide amino acid sequence (47-57), and then it was cloned in the prokaryotic host. Through optimizing the expression conditions, about 60% of the produced recombinant proteins were transferred to solution phase. Afterwards, the protein was purified using nickel column chromatography (Ni-NTA). A large extent of the recombinant proteins from a foreign source is accumulated in the bacterial host as inclusions with unusual folding.

If the produced recombinant protein is functional (e.g., enzymes), it is necessary to restore normal protein folding in order to regain its functionality. A common method to solve the aforementioned problem is to somehow reduce the production rate of recombinant proteins in host cells, so that the bacteria have more time for proper and normal folding of the foreign proteins. In some studies, the temperature of the induced culture was decreased (30˚C), which resulted in producing protein solution (17,18). In the present study, lower incubation temperature (18˚C) produced a greater amount of protein solution. In addition, 0.25 µM $ZnCl_2$ was added to the culture medium as mineral element. According to our findings, this element can contribute to maintaining the

natural folding and tertiary structure of proteins (19, 20). In a study of Jensen and colleagues, the sequence of the translocation domain of botulinum toxin (amino acids 449-552) was added to the end of light chain sequence to close the recombinant protein structure to the natural form. However, they did not obtain high yields of the recombinant protein with natural folding. They showed that incubation at lower temperatures could increase solubility of recombinant proteins. Hence, complete structure of a protein does not necessarily lead to producing natural folding proteins; rather, it can result in lower ability of the host bacteria in natural protein folding due to large recombinant protein sequence (18).

In this study, catalytic domain of BoNT/A was expressed as recombinant. The presence of cross-linking domain of toxin can help maintain the strength and resistance of the protein in natural systems. However, according to former studies, the immune system response against light chain botulinum toxin on its own is much less than the time it is linked with joint and cross-linking domains (18, 21). Therefore, the possibility of allergic and inflammatory reactions towards the recombinant protein produced in this study is very low. In addition, use of CPP is one of the least aggressive methods for transferring biomolecules into a variety of cell lines. In-vivo and in-vitro studies have shown that these peptides have very low toxic effects on their target cells (17). By producing recombinant protein solutions, there was no need for denatured purification or adding protein soluble compounds such as urea. This method reduces the duration of purification process and resolves the need for adjusting protein folding to its native state. Moreover, in this method, protein yield does not change and recombinant proteins maintain their functionality. The results of the current study indicate high expression of recombinant type A botulinum toxin light chain under optimized conditions.

In addition, according to the study results, protein purification through chromatography over a nickel-containing resin seems to be a very convenient and effective method for purification of histidine-tagged proteins.

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