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Original Article

Immunogenicity Evaluation of a Recombinant C-terminus of Botulinum Neurotoxin Type A Binding Domain Protein

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Abstract

Introduction: Botulinum neurotoxin is one of the most potent toxins. This neurotoxin is a biological weapon due to its high lethality and simplicity of preparation. The receptor-binding domain of botulinum neurotoxin is a promising vaccine candidate. In this study, the immunogenicity of the C-terminal domain of botulinum neurotoxin type A binding domain was investigated.

Materials and Methods: The synthetic gene encoding 285 C-terminal amino acids of BoNT/A binding domain fused to *trxA* gene, was constructed. The sequence was optimized codon usage for expression in *E. coli* and subcloned into pET-17b expression vector. The recombinant protein was expressed using 1 mM IPTG and purified by affinity chromatography on a column of Ni-NTA. The recombinant protein was confirmed by Western blotting and was used to immunize mice. The indirect ELISA and t-test were used to assess and compare antibody titers against recombinant protein.

Results: The codon adaptation index of the contract was altered from 0.62 to 0.90 after optimization. The minimum energy of the predicted mRNA structure was (-308.39) kcal/mol. SDS-PAGE and Western blotting confirmed the 44/6 kDa recombinant protein. Following immunization, mice elicited significant IgG antibodies in serum compared to control mice (p<0.05).

Conclusions: The results indicated a highly expressed and purified recombinant protein, which is able to evoke high antibody titers in mice. Future studies may develop the recombinant antigen as a potential immunogenic candidate against botulinum neurotoxin type A.

Keywords: *Clostridium botulinum*, Botulinum Neurotoxin type A, Binding Domain, Recombinant Protein, Immunogenicity

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Introduction

Botulism is a severe and potentially lethal form of food poisoning that results from the consumption of food contaminated with botulinum toxins (BoNTs). The first documented case of botulism occurred in 1735, and the initial outbreak took place in Germany, where consumption of contaminated sausages led to approximately 50% of the deaths. In the 19th century, the term "botulism" was coined from the Latin word "sausage" to describe the disease caused by eating contaminated sausages.[1] The illness is caused by bacterial toxins that interfere with nerve endings, resulting in paralysis, respiratory failure, and eventual death by impeding nerve impulse transmission. The toxins can enter the body via several routes, including the gastrointestinal tract, eyes, or respiratory tract. Upon entering the bloodstream or lymph system, they reach the nerve endings and cause paralysis.[2]

All strains of *Clostridium botulinum* produce botulinum toxins (BoNTs). The BoNTs are immunologically diverse and classified into seven serotypes denoted by letters A to G.[3] Among these, types A, B, E, and, on rare occasions, F, are responsible for botulism in humans. Serotypes A and B are typically associated with soil, while type E is mainly found in aquatic environments, including water, fish, and seafloor sediments.[4] Botulinum neurotoxin is one of the most potent toxins known, with estimated 50% lethal dose (LD_{50}) values of 1 ng/kg body weight.[5]

Botulinum toxin serotype A (BoNT/A) is a large protein with an average length of 1296 amino acids. The toxin is gradually released into the environment as a result of

bacterial growth and membrane destruction. BoNT/A is synthesized in the cytoplasm of the bacterial cell, and a portion of it is secreted into the extracellular space through the membrane.[6,7] The neurotoxins are 150-kDa proteins, consisting of a 100-kDa heavy chain (Hc) joined to a 50-kDa light chain (Lc) via a disulfide bond. The C-terminal of the heavy chain (HcC) (50 kDa) mediates binding of the neurotoxin to specific neuronal receptors, while the N-terminal (HcN) (50 kDa) allows the catalytically active light chain (LC) to enter the cytosol from late endosomes.[8] The heavy chain domain (HC) is the most widely studied structural domain of the neurotoxin and has been investigated for therapeutic purposes, primarily as an antigen for active immunization, as well as a carrier for intracellular transport of light chain inhibitors.[9] For instance, a recombinant botulinum vaccine containing the HC fragments of botulinum neurotoxins A and B, produced by the DynPort Vaccine Company for the U.S. Department of Defense, is presently undergoing clinical trials.[10]

Antibiotic treatment is not recommended for botulism, as it can lead to the release of more toxin upon bacterial cell death and lysis. The primary treatment for botulism is immediate medical care and administration of equine antitoxin. Passive immunization with antitoxin can prevent further nerve damage and reduce the severity of the disease if administered early. However, it cannot reverse the effects of paralysis and may cause side effects such as headaches, chills, nausea, vomiting, or weakness.[11,12]

Botulism is not a communicable disease, and there have been no widespread epidemics reported. Nevertheless, due to its high toxicity and ease of production, the Centers for Disease Control (CDC) classifies BoNT as a Class A bioterrorism agent.[13] The aim of this study was to express high levels of the HcC fragment of BoNT/A in *E. coli* BL21 (DE3) and to evaluate the immunogenicity of the recombinant protein as a potential candidate for active immunization against botulism. By achieving this aim, we hope to contribute to the development of effective strategies for preventing and controlling botulism, particularly in the context of bioterrorism threats.

Materials and Methods

In Silico Design of the Construct

The nucleotide sequence encoding the 285 C-terminal amino acids of BoNT/A binding domain (GenBank accession number NC_009495.1) and the trxA gene with a C-terminal 6x histidine tag was designed for the bioinformatics study (Supplementary Fig. 1). *NdeI* and *BamHI* restriction enzyme cleavage sites were inserted at the 3' and 5' ends of the nucleotide sequence, respectively. The Genscript Optimization GeneTM algorithm server (https://www.genscript.com/gene-optimization.html) was utilized to optimize the nucleotide sequence for the bacterial host. The Genscript™ Codon Optimization algorithm employs a multifactor approach, screening and validating over 200 factors involved in gene expression, including GC content, codon usage and content index, RNase splicing sites, and cis-acting mRNA destabilizing motifs. The second mRNA structure's thermodynamic analysis and prediction were carried out using the MFOLD server (http://unafold.rna.albany.edu/?q=mfold),[14] which predicts structures based on a thermodynamic model (the Gibbs free energy) and takes into account many parameters, such as pH, temperature, and the local composition bias of RNA, that affect folding. We used the Expasy ProtParam program (https://web.expasy.org/protparam/) to determine the physicochemical characteristics and stability of the recombinant proteins.[15] Finally, the EMBOSS Backtranseq tool (https://www.ebi.ac.uk/Tools/st/emboss_backtranseq/) was utilized to translate the protein sequence into the nucleotide sequence, and Shinegene was tasked with synthesizing it (China).

Transformation of the Recombinant Vector

For plasmid transformation, chemically competent *E. coli* BL21 (DE3) cells were mixed with 5 µL of pET17b-BoNT/A-HcC and incubated on ice for 30 min. Next, the cells were given a heat shock by placing the reaction mix at 42 °C for 30 s in a water bath, followed by incubation on ice. The transformed cells were then added to LB media and incubated at 37 °C with 140 rpm agitation for 3 h. After incubation, the cells were pelleted at 5000 rpm for 6 min and the supernatant was decanted. A solid Luria-Bertani (LB) culture medium containing ampicillin at 200 µg/ml was used to screen for plasmid-containing cells. Plasmid extraction was performed using the alkaline lysis method to confirm the construct. After plasmid extraction, PCR was performed using universal primers specific for the pET-17b plasmid (T7 promoter primer) to verify the presence of the BoNT/A-HcC gene. The PCR program consisted of one cycle of initial denaturation at 93 °C for 3 min, followed by 35 cycles of denaturation at 93 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 90 s, and a final extension cycle at 72 °C for 5 min. The results were evaluated on a 1% agarose gel. In addition, recombinant clones were confirmed by restriction digestion using two restriction enzymes, *NdeI* and *BamHI*, and the resulting enzyme cleavage sample was analyzed on a 1% agarose gel.

Expression and Purification of the Recombinant Protein

As a bright passage for protein expression, an overnight culture of recombinant *E. coli* BL21 (DE3) was used to inoculate 10 mL of LB medium. Protein expression was induced by adding IPTG (Isopropyl-D-thiogalactopyranoside) at a final concentration of 1 mM when the culture reached an optical density of 0.7 (OD600). After 5 hours, cells were collected by centrifugation at 3000 g for 5 min. The bacterial

residue was then mixed with lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH = 8) and sonicated 6 times at high power for 10 s. The cell extract was clarified by centrifugation at 10,000 g for 15 min, and protein expression was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

For mass expression, the same procedure was repeated in a volume of 1 liter. The cell extract was loaded onto a Ni-NTA column affinity chromatography system (ArgPure, Iran), and the column was washed with 10 column volumes (CV) of binding buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, pH = 8). The protein was eluted from the column with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, and 500 mM imidazole, pH = 7.4), and the pure protein was dialyzed against elution buffer without imidazole.

Western Blot Analysis

The chimeric protein was electrophoretically transferred onto a nitrocellulose membrane (Roche) using transfer buffer (39 mM glycine, 48 mM Tris-base, 0.037% SDS, and 20% methanol). After transfer, the membrane was blocked with 5% skimmed milk and subsequently washed with PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 0.05% (v/v) Tween 20). The membrane was then incubated with a 1:10,000 dilution of mouse anti-his-tag IgG (sima) at 37 °C for 1 h. The protein of interest was detected by 3,3´-diaminobenzidine (DAB) as a chromogenic substrate, and the reaction was stopped by washing with distilled water.

Animal Immunization

Female Bagg Albino (BALB/c) mice aged 6 to 8 weeks were utilized in the vaccination trials. The animals were maintained under standard hygienic conditions at the Animal Care Facility of Imam Hussein University. The mice were divided into three groups, namely, control, free rBoNT/A-HcC, and rBoNT/A-HcC with adjuvant. Subcutaneous injections were administered to each group between the skin and the muscle, with each group receiving 20 µg of recombinant BoNT/A-HcC either alone or in combination with Freund's adjuvant. The administration was performed four times with 7-day intervals. One week after each treatment, blood was collected through retroorbital blood sampling. The sera were obtained by centrifuging the blood at 800 g for 15 min and stored at -20 °C for further analysis.

Antibody Response

To assess the IgG antibody response, we employed the enzyme-linked immunosorbent assay (ELISA) test. Specifically, Maxisorp plates from Nunc were coated with 5 µg of recombinant BoNT/A-HcC protein (in 100 µl of coating buffer) overnight at 4 °C. After triple washing, the plates were blocked with 5% skim milk in PBST (PBS + 0.05% Tween 20) for 1 h at 37 °C. Subsequently, serial dilutions of the serum samples were added to the plates and incubated for 1 h at 37 °C. The plates were then washed with PBST and incubated with 100 µl of 1/5000 Goat Anti-Mouse IgG H&L (HRP) (ab205719) for an additional hour at 37 °C. After another round of washing with PBST, 100 µl of the substrate solution, containing o-phenylene diamine, was added to each well. Finally, the reaction was stopped with 2.5M H_2SO_4 and the absorbance of the wells was measured at 495 nm on a microplate reader.

Statistical Analysis

In the present study, all statistical analyses were conducted using the SPSS software. Normality of the data was determined using the Kolmogorov-Smirnov test. The t-test was used for the data analysis of variance and comparison, with a significance level of $p<0.05$ considered as statistically significant. The statistical analysis was performed for the second, third, and fourth injections.

Results

Bioinformatics Analysis

The sequence encoding 285 C-terminal amino acids of BoNT/A binding domain and *trxA* gene with a C-terminal 6x histidine tag was constructed for the bioinformatics study. The Expasy server was used to investigate the main physicochemical features. The molecular weight and isoelectric point are calculated discretely at 44.6 kDa and 9.16, respectively. The protein had an aliphatic index of 91.24 and an instability index of 30.88. The overall number of positively and negatively charged amino acids was 52 and 41, respectively. Protein-sol calculated that it is a soluble protein (0.894) upon overexpression using the default threshold (0.4). The DISULFIND webserver also rejected disulfide bond formation.

Through efficient optimization, the prokaryotic host's codon adaption Index (CAI) was increased from 0.64 to 0.90 and the GC content, which is required for transcription and translation operations, increased from 30.92% to 43.88% (Figure 1).

According to the MFOLD results, mRNA had high stability and no large loops in section 5' (Figure 2). After using NEB cutter V2.0 to check the restriction enzyme cut sites, the cut sites of two restriction enzymes, *Ndel* and *BamHI*, were placed at the 3' and 5' ends of the construct and sent to Shinegene Company for ordering.

Expression and Purification of the Recombinant Proteins

Following cassette design, restriction endonucleases were used to digest the pET-17b plasmid according to MCS. The integrity of the recombinant plasmid was confirmed using restriction enzyme double digestion and PCR process using T7 universal primer. The PCR product showed a fragment of

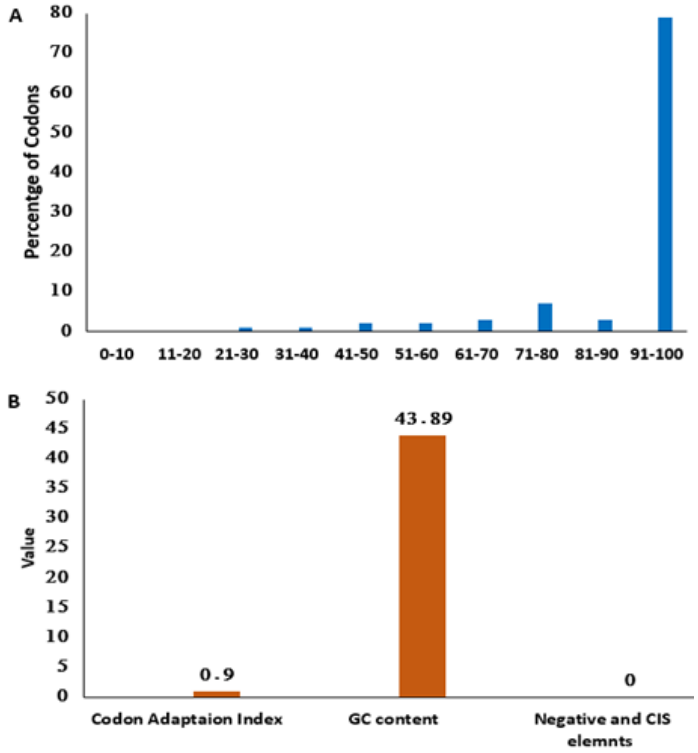

Figure 1. Codon Usage Bias Adjustment. **(A)** The percentage distribution of codons in computed codon quality groups. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism. **(B)** The codon adaptation index (CAI) and the GC count of the gene after optimization.

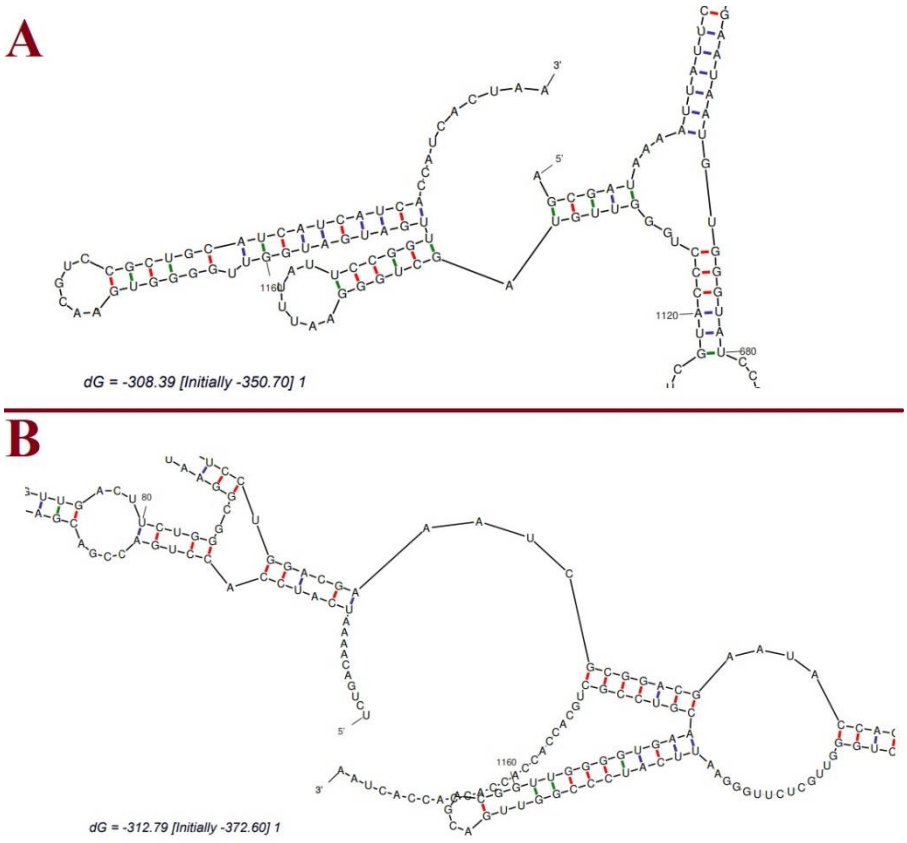

Figure 2. mRNA Structure after **(A)** and before **(B)** Optimization Predicted by the MFOLD Server. The predicted structure after optimization (A) does not have long stable hairpin and pseudoknots at the 5′ sites of mRNA. In addition to the amount of free energy, these characteristics are useful for optimal expression.

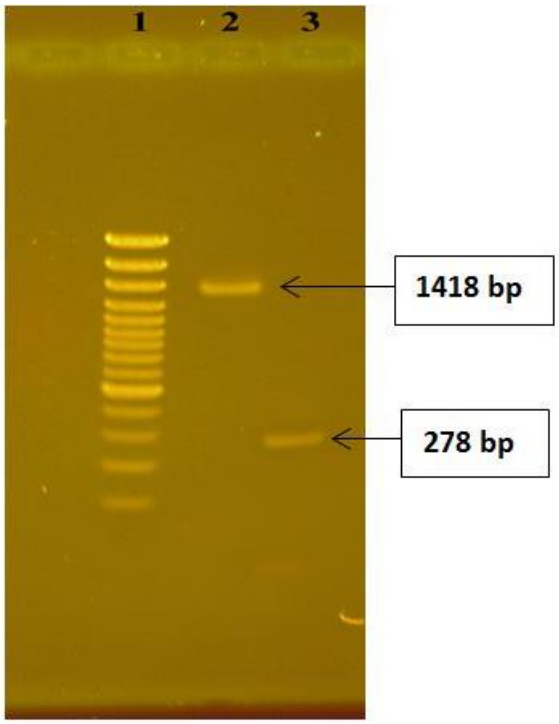

Figure 3. BoNT/A-HcC was PCR amplified using the t7 universal primers and as detailed in the text. Lane 1: 100 bp DNA ladder plus; Lane 2: PCR product of the recombinant pET17b-BoNT/A-HcC plasmid; Lane 3: PCR product of the pET-17b plasmid without insert.

approximately 1418 bp (Figure 3). The restriction enzyme analysis revealed that the 1200 bp target gene was correctly inserted into the recombinant plasmid (Figure 4).

The expression of recombinant protein was induced with 1 mM of IPTG. Protein band were observed on 12% SDS-PAGE and had a molecular weight of 44.6 kDa, which supports the findings of the molecular mass check with ProtParam server. When compared to induced or non-induced cells, the induced protein, as shown by the SDS-PAGE, has a unique band (Figure 5).

The protein was purified under non-denaturing conditions, and SDS-PAGE analysis revealed that the recombinant protein was present in the eluted fraction (Figure 6-A). Western blotting confirmed the purified protein's reaction with the anti-His-tag (Figure 6-B). The concentrations of the purified proteins were determined using a NanoDrop spectrophotometer after dialyzing them against PBS. The recombinant protein was obtained at a concentration of 0.85 mg/ml.

Determination of Serum IgG Titer

To assess the humoral immune response, specific antibodies to recombinant protein were measured. Following vaccination, the animals remained healthy and showed no signs of abnormal behavior. The ELISA results revealed that the sera

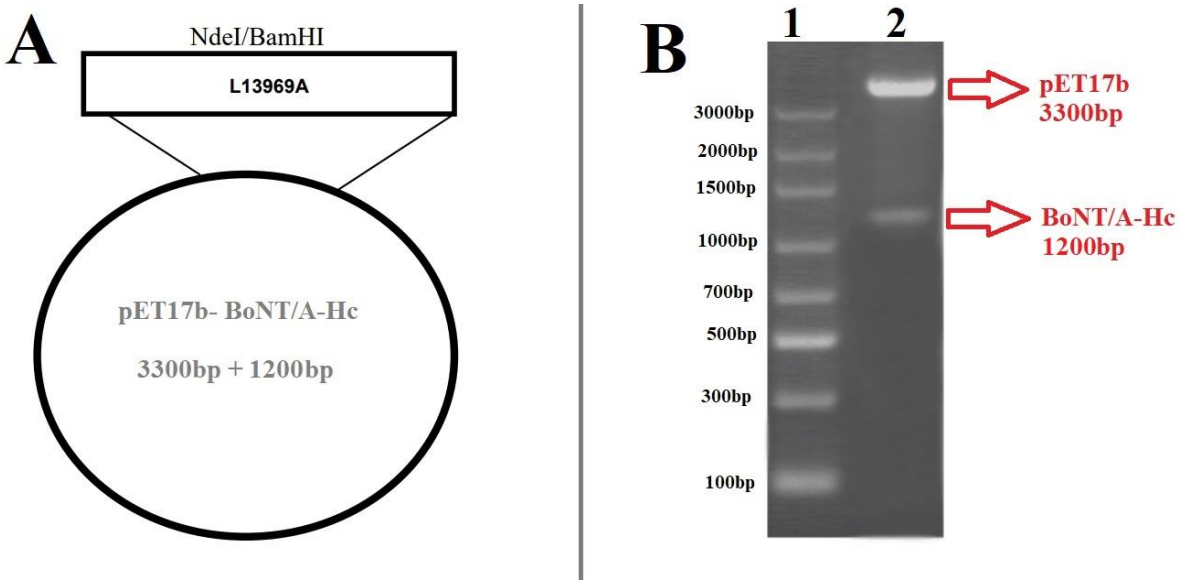

Figure 4. **(A)** Schematic Design of pET17b-BoNT/A-HcC. **(B)** Verification of Recombinant pET17b-BoNT/A-HcC through Enzyme Digestion. Lane 1: 1kb DNA ladder; Lane 2: pET17b-BoNT/A-HcC digested by two restriction enzymes (*NdeI* and *BamHI*).

of immunized mice had proper IgG titers against BoNT/A up to 1:12800 sera dilutions (Figure 7). The ELISA detected a low serum IgA titer, which did not differ statistically from the control group (p>0.05, data not shown). When mice were immunized, they produced significantly more IgG antibodies in their serum than control mice (p<0.05).

Discussion

Botulism is a severe and often fatal disease caused by one of the seven serotypes of *Clostridium botulinum*'s neurotoxin. The molecular mechanism of the toxin reveals that the light chain is responsible for the catalytic function, while the heavy chain comprises two domains: the transfer domain at

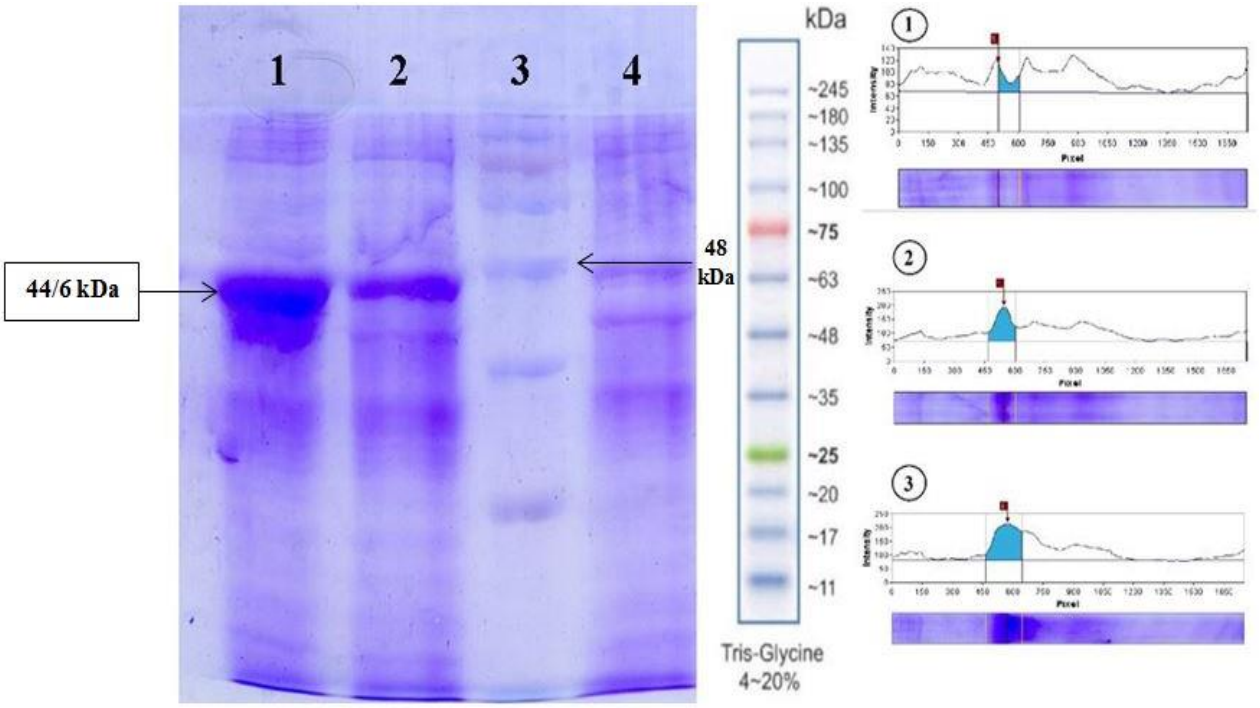

Figure 5. SDS-PAGE Analysis of Recombinant Protein Expression in *E. coli* BL21 (DE3). Lane 1: Cell lysate fractions from 1 mM IPTG-induced bacterial culture; Lane 2: Cell lysate fractions from 0/5 mM IPTG-induced bacterial culture; Lane 3: Prestained Protein Ladder (PR901641); Lane4: Cell lysate fractions from un-induced bacterial culture.

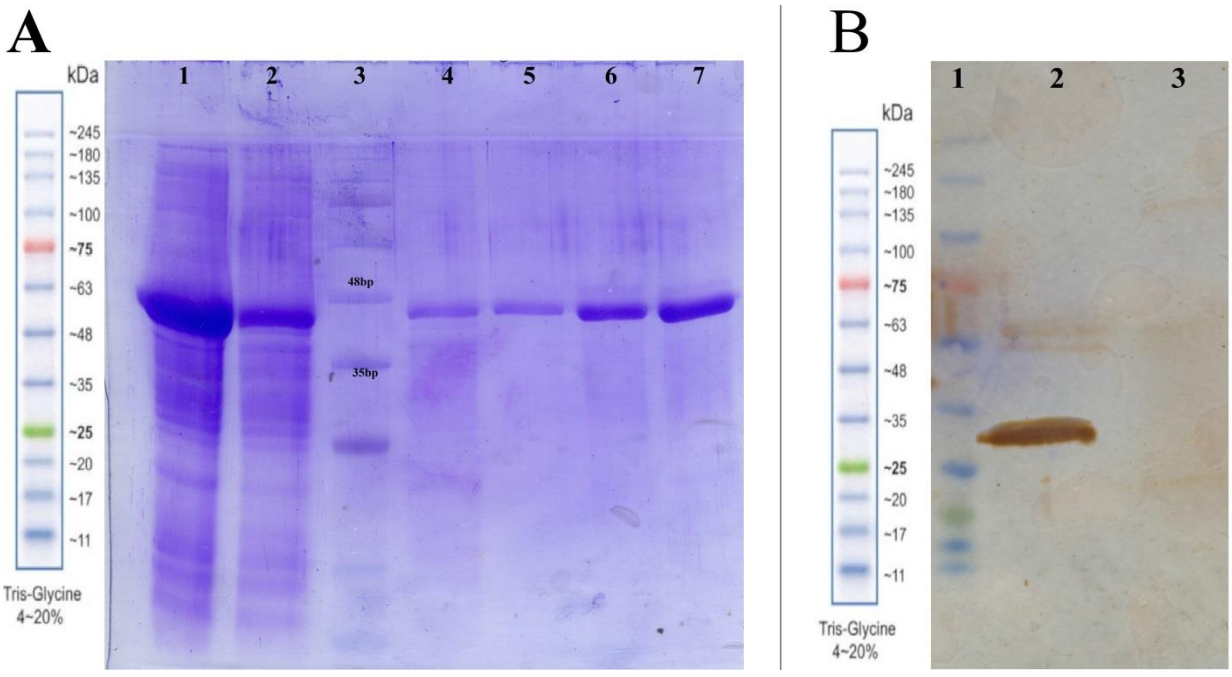

Figure 6. Analysis of Purified Recombinant BoNT/A-HcC Protein by SDS-PAGE **(A)** and Western Blot **(B)**. **(A)** Lane 1: recombinant protein before loading on the column; Lane 2: flow-through (after applying the cell lysate to the column); Lane 3: Prestained Protein Ladder (PR901641); Lanes 4 & 5: washing the column with 20 mM imidazole buffer; Lanes 6 & 7: pure protein eluted by 450 mM imidazole buffer. **(B)** Lane 1: Prestained Protein Ladder (PR901641); Lane 2: pure recombinant protein BoNT /A-HcC; Lane 3: BSA (negative control).

the N-terminus and the binding domain at the C-terminus. Neutralizing botulinum neurotoxin involves binding it to the neuromuscular synapse using antibodies against the Hc domain. Previous studies have demonstrated that a recombinant fragment of the carboxyl terminus of the heavy chain of tetanus toxin, which shares high homology with botulinum toxin, can protect mice from a 10^2 dose of the toxin's LD_{50}.[16] This method has also been employed to develop new vaccines

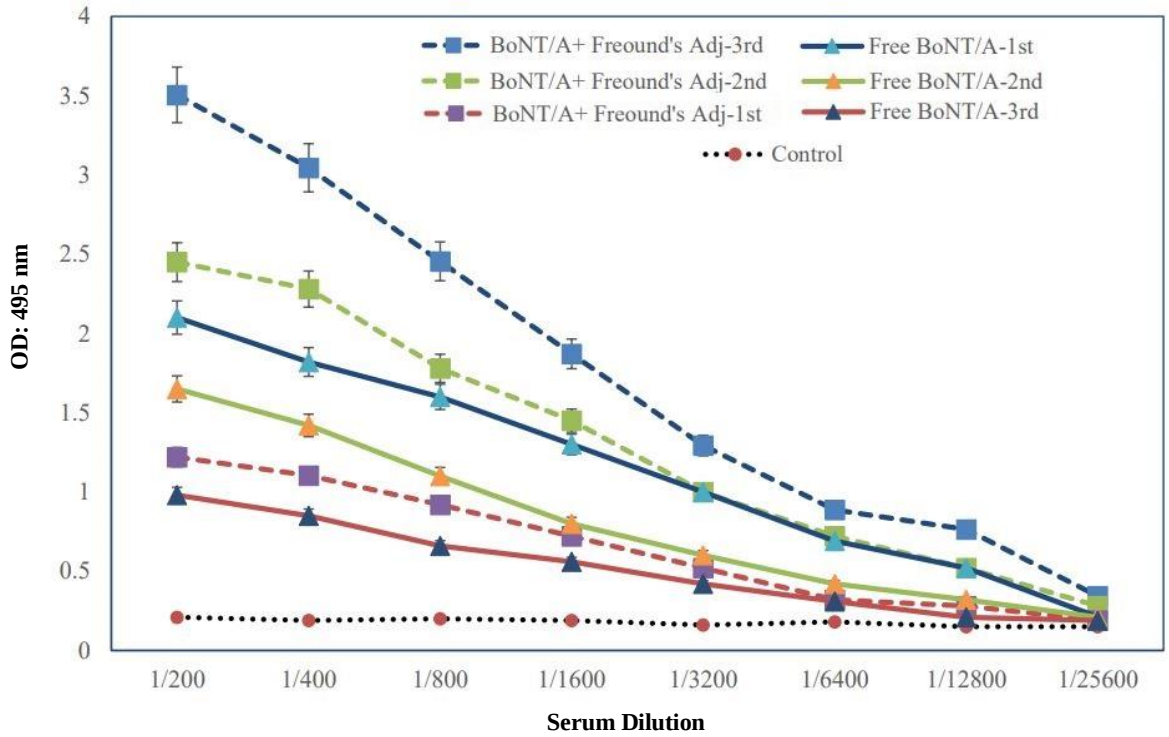

Figure 7. Serum Antibody Titer Investigation in Mice Immunized with the Recombinant Immunogen by ELISA. The graphs show the ELISA-detected IgG titer of blood samples taken one week after each treatment. There was no statistically significant difference between the mice received the recombinant immunogen with or without Freund's adjuvant. However, there was a significant difference between the immunized and control groups.

against botulinum toxin due to the structural and sequence similarities of the neurotoxins produced by *Clostridium tetani* and *Clostridium botulinum*.[17]

It is crucial to develop effective vaccines against botulinum toxin since there is no cure for botulism, and the disease can be fatal if left untreated. The current treatment options involve supportive care and administration of antitoxin, but the antitoxin is only effective if administered early in the disease course before irreversible damage has occurred. Therefore, the development of effective vaccines is of paramount importance in preventing botulism.

To combat botulism, toxoid vaccines are widely used worldwide. However, due to the disadvantages associated with toxoid vaccines, recent efforts have been focused on producing recombinant vaccines.[18] The drawbacks of these vaccines include the need for specialized facilities and equipment, which raises the price, the inefficiency of *Clostridium botulinum*'s toxin production, the dangers associated with handling botulinum toxin, etc.[19] Due to the problems mentioned, today they are turning to recombinant vaccines. Numerous studies on the production of recombinant vaccines and antibodies have focused on the toxin-binding domain. One of these vaccines is recombinant proteins derived from the binding domain, and antibodies against this recombinant protein can counteract the effects of botulinum type B neurotoxin.[20]

In bioinformatics analyses, using thermodynamics in the prediction algorithm of RNA structure is not common with Single sequence secondary structure prediction software. Up till now, only three programs, (Mfold, RNA fold, and RNA shapes) is known to use this feature.[21] In this study, we used the Mfold program as a tool to predict the secondary structure of RNA.[22] This server has the advantages of being very easy to use and free online. In addition, Mfold is an Internet-based program that runs on (almost) any computer with Internet access, has many, versions, and is regularly updated. Mfold can predict the energetically optimal secondary structure of an RNA molecule by using of Zuker algorithm. In this study, the Gibbs free energy before and after sequence optimization was -308 and -312, respectively, which confirms the stability of mRNA. Codon usage plays a crucial role when recombinant proteins are expressed in different organisms. This is especially the case if the codon usage frequency of the organism of origin and the target host organism differ significantly. Therefore, to enhance efficient gene expression it is of great importance to identify rare codons in any given DNA sequence and subsequently mutate these to codons which are more frequently used in the expression host.[23] In this study, GenScript's codon optimization tool was utilized to improve the expression sequence. Finally, after optimizing the sequence for expression in the prokaryotic host, the amount of CAI was closer to 1 that considered to be ideal (The lower number, the gene will be expressed poorly).

In this study, after expressing part of the C-terminal fragment of receptor binding domain, the amount of antibody titer in the laboratory animal was evaluated. In the first step, a smaller protein of the carboxyl terminus of the botulinum type A gene was expressed in the *E. coli* host, while in several studies, the complete toxin molecule has been used as an immunogenic antigen.[24,25] The pET vector with T7 lac promoter was used to express the recombinant antigen. Another feature of this vector is the presence of the 6 His-Tag on both sides of the MCS, which facilitates the purification of the expressed recombinant protein. The ELISA method has been used to demonstrate the immunogenicity of recombinant protein. According to the ELISA results, the neutralizing antibody titer after the fourth injection indicates a complete body response to the prescribed antigen. Statistical analyzes on test and control samples by t-test confirmed this hypothesis.

Today, genetic engineering and recombinant production of various components of botulinum neurotoxins, especially the binding region, and their introduction as candidates for recombinant vaccines continue rapidly. In 1995, the type A binding site was cloned and its prophylactic effect was confirmed in mice.[26] In 2004, the neurotoxin binding site of type B was cloned and its binding properties were investigated.[20] The Hc region in all botulinum serotypes in *E. coli* is individually cloned and expressed and is currently in the clinical trials as botulinum vaccine candidates.[19] Numerous studies have been performed on the expression of the Hc-C subunit of the botulinum type A neurotoxin.[25] Some attempts to express this product in *E. coli* resulted in poor yields due to the low solubility of the recombinant product.[24] In this study, C-terminal domain of the C-terminal fragment of the heavy chain botulinum type A neurotoxin was expressed and purified. The results of antibody titers in this study were the same as other studies that had selected a larger part (with a molecular weight of 100 kDa or 55 kDa) of this region.

Conclusion

In conclusion, we have successfully developed a recombinant protein based on the heavy chain of botulinum neurotoxin type A that is immunogenic in mice. This study provides a promising strategy for the development of effective vaccines against botulinum toxin, which can have significant implications in preventing and treating botulism. Further studies are needed to evaluate the safety and efficacy of the recombinant protein in humans.

Authors' Contributions

The authors contributed equally to this study.

Ethical Approval

For animal experiments, suitable precautions were taken to reduce the animals' suffering and distress, and all experimental techniques followed the ethical norms for handling with animals.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgment

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