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Fusion and cloning of the binding domains of botulinum neurotoxin type A and B in *E. coli* DH5α

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ABSTRACT

Botulinum neurotoxins (BoNTs) are the most potent bacterial toxins that cause paralysis at femtomolar concentrations by blocking acetylcholine neurotransmitter release. Each BoNT is composed of a light chain (50 kDa) which acts as a metalloprotease, and a heavy chain (100 kDa). The carboxyl-terminal domain (HC) of heavy chain mediates binding to the specific cell surface receptors and has a considerable immunogenicity without any significant toxicity. In this study the binding domains of BoNT/A,B were joined together to produce a recombinant chimeric protein as a vaccine candidate. The relative genes were synthesized in pET28a(+) expression vector separately then appropriate primers were designed to amplify the binding domain gene of BoNT/B. The restriction sites for NcoI and NdeI endonuclease were placed at 5' end of forward and reverse primers respectively. The PCR products then were digested with NcoI and NdeI enzymes. At the same time the construct of pET28a (+) and binding domain A gene was digested with the same enzymes. Digested fragments were joined together by T4 DNA ligase. The recombinant construct then introduced into E.coli DH5α. Our enzymatic digestion, PCR amplification and sequencing of recombinant construct confirmed the correct fusion of BoNT/B-HC and BoNT/A-HC genes to produce a chimeric gene.

Keywords: Botulinum neurotoxin, binding domain, chimeric gene, chimeric vaccine

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INTRODUCTION

Botulinum neurotoxins (BoNT) produced by *clostridium botulinum* are the most potent toxins in human and they are agents responsible for the deadly food poisoning disease known as botulism [1]. *Clostridium botulinum* produce seven serotypes of BoNTs named as A to G. Botulinum neurotoxins act principally on the peripheral nervous system where they inhibit release of acetylcholine at the neuromuscular junction caused a widespread descending flaccid paralysis and ultimately the botulism syndrome [2].

BoNTs are expressed as single polypeptide chain with the molecular weight of about 150KDa cleaved endogenously or exogenously resulting in a 100KDa heavy chain(HC) and a 50 KDa light chain(LC) linked with a disulfide bond [1]. C-terminal and N-terminal domain of the heavy chain are the binding and translocation domain respectively [4]. The C-terminal of heavy chain is responsible for neuro-specific binding at the presynaptic membrane of the neuromuscular junction [4,5]. The N-terminal of heavy chain plays critical role in translocation of the catalytic light chain into the cytosol [6].

Because of extreme toxicity and food poisoning, producing an appropriate vaccine against botulism is necessary. Immunization against these neurotoxins is achieved by the injection of botulinum neurotoxins inactivated by formaldehyde(toxoids) [7] however toxoids have side effects such as allergic responses made production of new vaccines necessary[3,8,9,10]. Recombinant vaccines is one of these efforts[7]. Using recombonant DNA technology, we can produce efficient and pure antigens without growing and handling the *Clostridium botulinum* bacteria[11]. At present different domains of botulinum neurotoxins are produced by recombonant DNA technology and their immunogenicity are evaluated[12-33]. Binding domains from A-F types produced as recombinant protein up to now and evaluated for a vaccine candidate [22-33].

The results of these investigations represent binding domain's high potential for botulism prevention [22-33]. Moreover the effective bivalent recombinant vaccine can be produced from mixture of binding domains of A and B types [11]. In the botulinum chimeric vaccine field designing and producing recombinant chimeric antigen consisting of binding domains from A, B and E types (common human pathogenic organisms) and determining their antigenic properties investigated [34]. Researches indicate that using small fragment of neurotoxin binding domain could not cause effective immunity[12] so in this study for achieving effective immunity, whole length of binding domains was used.

MATERIALS AND METHODS

2-1.Optimization and gene synthesis

Gene sequences for binding domains was obtained from gene bank (accession numbers: EF470981 and AF295928) then A+T, C+G contents, rare codons ,and mRNA stability were optimized. Each of these genes(*NdeI* and *XhoI* fragments) were synthesized and cloned in pET28a(+) expression vectors by the shinegene company.

2-2.Type B neurotoxin binding domain amplification

To fuse these tow genes, after plasmid extraction the type B neurotoxin binding gene was amplified by PCR. A pair of Primers (5'AATATAAATTTACCATGGTGCCGTTTGACCTTTC3') which adds a *NcoI* site and (5'AAATAAAAAAATCATATGTTCGGTCCAACCTTCGT3') which adds a *NdeI* restriction site to the 5' end was designed. PCR was performed in 25 µl total volume containing *Pfu* DNA polymerase (2 units) and its buffer, 0.2 mM of each dNTP, 10 pmol of both primers, and 16 µg Plasmid and $MgSo_4$ (4 mM). Samples were preheated for 5 min at 95°C and then 34 cycles of PCR was done: 30 second at 95°C, 50 second at 68°C, and 1.5 minute at 72°C. After the last cycle, the reaction was continued for additional time (5 min at 74°C). The size of amplified DNA fragment was analysed by 1.0% agarose gel.

2-3. Enzymatic digestion of BoNT/B binding domain gene (BoNT/B-HC) amplified by PCR and pET28a (+)-BoNT/A-HC

Type B neurotoxin binding domain gene was inserted into pET28a (+)-BoNT/A-HC recombinant vector between *NcoI* and *NdeI* restriction sites adjacent type A neurotoxin binding domain gene. Amplified BoNT/B-HC gene was extracted from gel by PCR product extraction kit (Bioneer, south Korea) then was digested with *NdeI* (*NdeI* 30 unit,1X orange buffer,360 ng PCR product, 4 hour) and *NcoI* (60 unit, 1X buffer, 360 ng PCR product, 12 hour). In the next step enzymes and other reaction components were removed by extracting digested products from the agarose gel. In the same way, recombinant pET28a(+)-BoNT/A-HC vector was digested with *NdeI* (recombinant plasmid 175 ng, *NdeI* 30 unit,1X buffer, 1 hour) and *NcoI* (recombinant plasmid 175 ng, *NcoI* 30 unit,1X buffer, 12 hour) and then extracted from the gel.

2-4. Ligation reaction between BoNT/B-HC gene and pET28a(+)-BoNT/A-HC recombinant vector and transforming the construct into *E.coli* DH5α competent cells

The concentration of purified and digested BoNT/B-HC and recombinant plasmid(**pET28a(+)-BoNT/A-HC**) was measured. Then by using 1:1 and 3:1 molar ratio of the Insert DNA-to- vectors, ligation reaction was accomplished in 16°C for 12 hour by T4 DNA ligase. After this process achieved recombinant construct transformed to E .coli DH5α competent cells using heat shock method for two minutes at 42°C. Transformed cells were grown for 12 hours at 37°C in McConkey medium supplemented with kanamycin (80µg/ml). The recombinant strains were grown in LB medium supplemented with kanamycin (80µg/ml) at 37°C until OD_{600}= 0.6 and then plasmid extraction reaction was carry out. PCR, enzymatic digestion and sequencing methods were used to confirm chimeric gene produced.

RESULTS

3-1. Gene optimization and synthesis

Codons of type A and B binding domain genes were optimized for E .coli by Optimum Gene™ algorithm. Following optimization, type A gene GC content increase from 23.72 to 43.82 percent (figure 1) and type B gene GC content increase from 23.02 to 41.86 percent (figure 2).

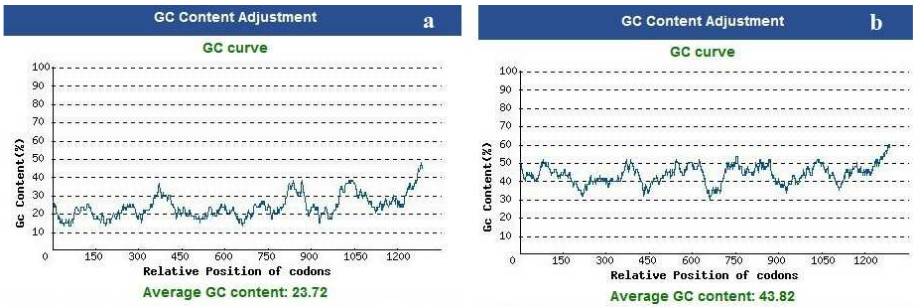

Figure 1: type A gene GC content before (a) and after (b) gene optimization.

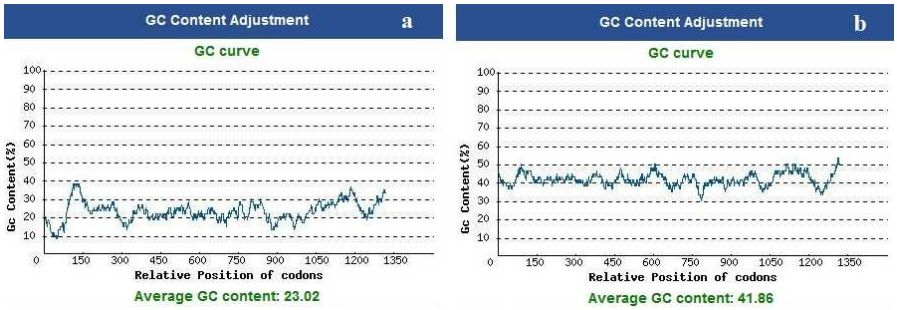

Figure 2: type B gene GC content before (a) and after (b) gene optimization.

CAI coefficient changes (the amount of similarity between codons of gene of inrerest and codons of genes which have high level of expression in *E.coli*) was shown in Pictures 3 and 4. After gene optimization, results showed that CAI coefficient increased.

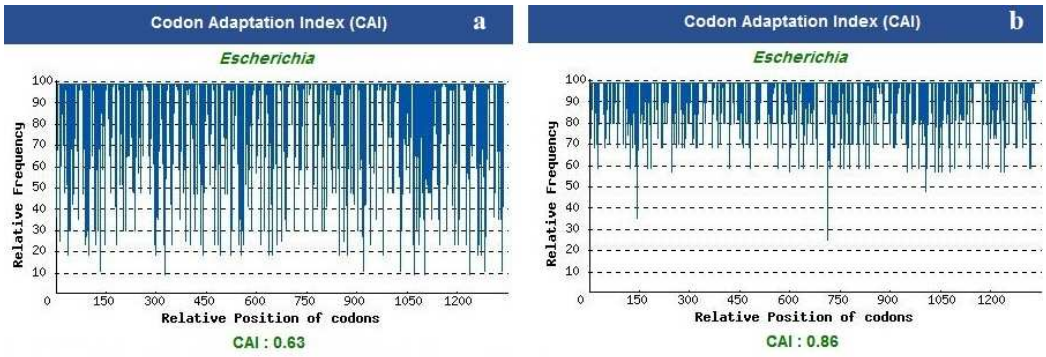

Figure 3: The CAI coefficient of type A neurotoxin binding domain gene before (a) and after (b) optimization.

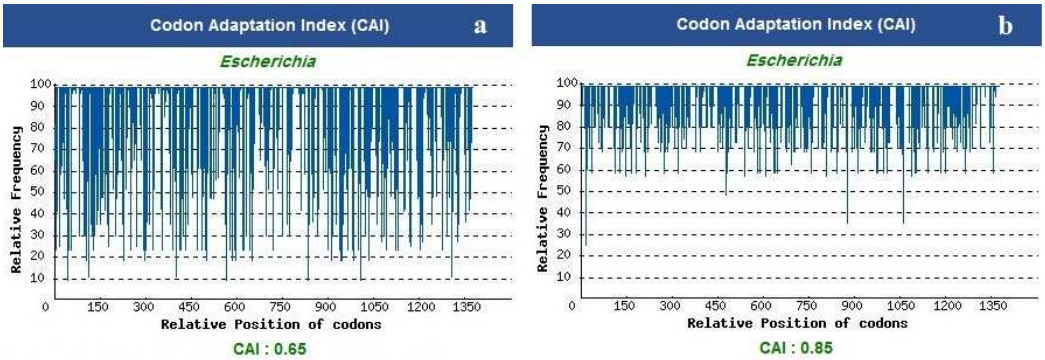

Figure 4: The CAI coefficient of type B neurotoxin binding domain gene before (a) and after (b) optimization.

The structure of naive and optimized mRNAs and their free energy modifications were analyzed by mfold software. Free energy modifications(Dg index) of the type A binding domain mRNA was -182 kcal/mol and after optimization it's Dg index was increased to -250 kcal/mol and Dg index for type B binding domain mRNA was increased from -207 kcal/mol to -235 kcal/mol after optimization therefore better conditions were expected for ribosome function (figure 5,6).

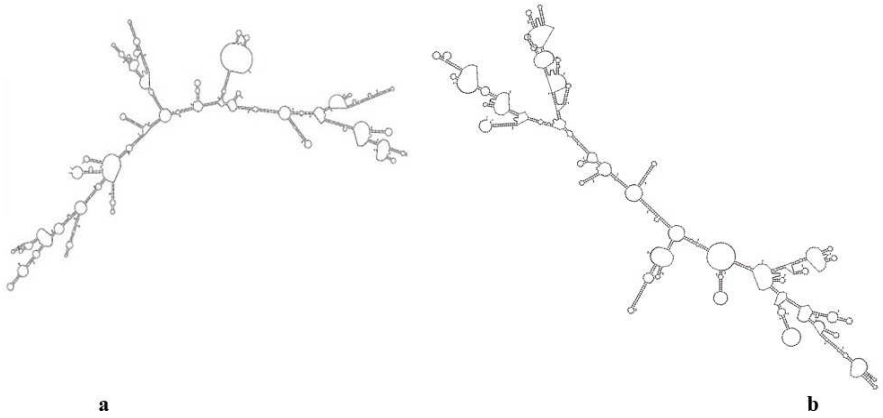

Figure 5: Secondary structure of type A gene mRNA before (a) and after (b) optimization.

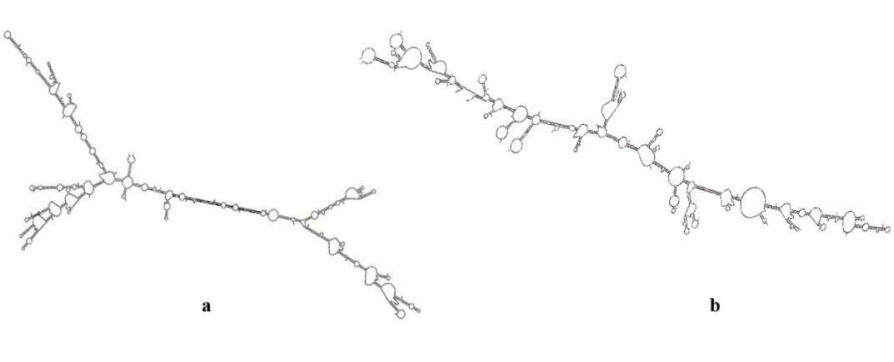

Figure 6: Secondary structure of type B gene mRNA before (a) and after (b) optimization.

3-2. Amplification of type B neurotoxin binding gene

DNA fragment of type B neurotoxin binding domain was amplified by forward and reverse primers which have *NcoI* and *NdeI* restriction sites. Pfu DNA polymerase enzyme which has proofreading ability was used for DNA amplification. The size of amplified DNA fragment was confirmed by 1.0% agarose gel electrophoresis (~1.4 kb).

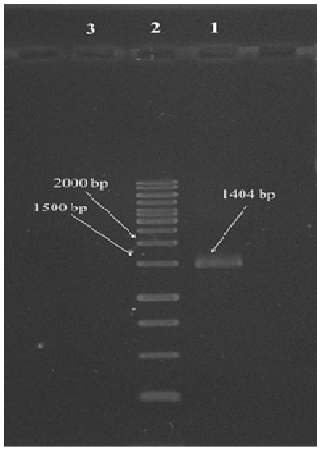

Figure 7: Elecrophorosis analysis (1.0%) of type B binding domain gene. Line 1: PCR product, Line 2: DNA marker, Line3: Negative control

3-3. Enzymatic digestion of BoNT/B binding domain gene and pET28a(+)-BoNT/A-HC

Purified PCR products and type A recombinant plasmid were digested by *NcoI* and *NdeI* restriction enzymes. Digested PCR products was analyzed by 12.0% native page electrophoresis and 13 and 14 bp DNA fragments was obtained (figure 8a) and recombinant plasmid digestion by restriction enzymes was confirmed by 1.0% agarose gel electrophoresis (figure 8b).

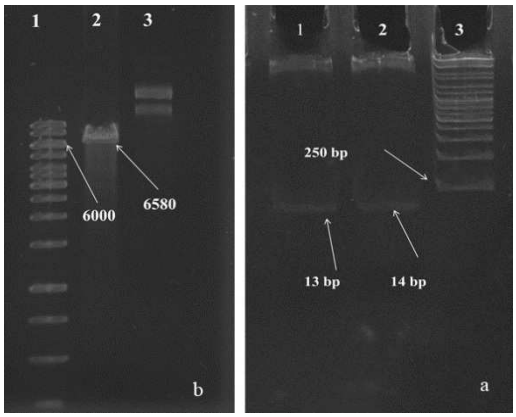

Figure 8: (a) Electrophoretic analysis of digested PCR products on 12.0% polyacrylamide gel. line 1: PCR products digested by *NcoI*, line2: PCR products digested by *NdeI*, line3: DNA marker, (b): Digested recombinant pET28a(+) plasmid analyzed on 1.0% gel. Line1: DNA marker, Line2: Digested recombinant plasmid, Line3: Intact recombinant plasmid.

3-4. Ligation reaction between BoNT/B binding domain gene and recombinant plasmid

Digested BoNT/B binding domain gene and recombinant plasmid was purified and extracted by gel elecrophorosis and then ligation reaction was done between this gene and recombinant vector. Achieved recombinant construct was transferred into *E.coli* Dh5α competent cells. Colony growth in medium containing kanamycin (80µg/ml), plasmid extraction from colonies, enzymatic digestion by *NcoI* and *XhoI* restriction enzymes and PCR reaction for type B binding domain gene confirm chimeric gene formation.

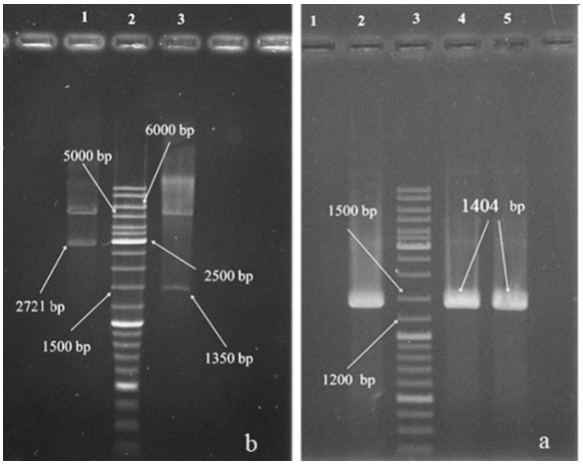

Figure 9: (a) Colony analysis by PCR technique: Line1: Colonies without chimeric gene, Line 2: Positive control, Line 3: DNA marker, Line 4,5: Colonies containing chimeric gene .1377 bp band indicate type B binding domain gene.(b) pET28a(+)-BoNT/A-HC recombinant vector in comparison with recombinant vector containing chimeric gene. Recombinant vector containing chimeric gene digested by *NcoI* and *XhoI*. Line 1: Double digestion of new recombinant plasmid and chimeric gene excision (2721 bp). Line2: DNA marker. Line 3 : Digestion of pET28a(+)-BoNT/A-HC recombinant vector by *NdeI* and *XhoI*.

DISCUSSION

The data obtained indicate that the binding domain can be cloned and expressed in *E. coli*. Recent works have also shown that binding domain gene derived from botulinum neurotoxins types A and B can be exploited as vaccine candidates against their respective toxin subtypes [9,26]. Development of a new recombinant vaccines could alleviate many of the problems associated with toxoids. Moreover using of genetic and protein engineering techniques, we can produce recombinant chimeric proteins as a recombinant vaccine.

Research carried out botulism prevention is more focused on production of recombinant vaccines. Neurotoxin light chain, inactive holotoxin and recombinant proteins comprise of translocation and catalytic domains produced as recombinant protein up to now [11,15,19-21,35]. The light chain can elicit protective immunity, but other domains (binding and translocation domains) of the toxins in comparison to light chain have high protective immunity power. Inactive holotoxins elicit high level of protective immunity, but their large size could make local and global unwanted reactions.

Recombinant proteins consist of translocation and catalytic domains have very different effective immunity dosage but the binding domain due to having effective epitopes and causing effective immunity is attractive goal for vaccine production.

In this research to achieve effective immunity, type A and B neurotoxin binding domains were fused as a first step for production of chimeric vaccine. Because the binding domain genes are sizable, fusion of these genes in the first step is a significant process. The partial parts of the HC domain have significantly less efficient protective immunity than the whole HC domain [2,12], therefore it was expected that using whole length of binding domains and forming a structure like to natural neurotoxin structure and also having entire effective epitopes, make recombinant protein to eliciting significant protective immunity achievable. Bivalent recombinant vaccine against type A and B (rBVA) that comprise, mixture of type A and B binding domains, have a effective immune efficiency, therefore it was expected that the resulted recombinant chimeric protein simultaneously have mentioned vaccine abilities and chimeric vaccine benefits.

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