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Comparative Study of Immunological and Structural Properties of Two Recombinant Vaccine Candidates against Botulinum Neurotoxin Type E

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ABSTRACT

Background: Recently, botulinum neurotoxin (BoNT)-derived recombinant proteins have been suggested as potential botulism vaccines. Here, with concentrating on BoNT type E (BoNT/E), we studied two of these binding domain-based recombinant proteins: a multivalent chimer protein, which is composed of BoNT serotypes A, B and E binding subdomains, and a monovalent recombinant protein, which contains 93 amino acid residues from recombinant C-terminal heavy chain of BoNT/E (rBoNT/E-HCC). Both proteins have an identical region (48 aa) that contains one of the most important BoNT/E epitopes (YLTHMRD sequence). **Methods:** The recombinant protein efficiency in antibody production, their structural differences, and their BoNT/E-epitope location were compared by using ELISA, circular dichroism, computational modeling, and hydrophobicity predictions. **Results:** Immunological studies indicated that the antibody yield against rBoNT/E-HCC was higher than chimer protein. Cross ELISA confirmed that the antibodies against the chimer protein recognized rBoNT/E-HCC more efficiently. However, both antibody groups (anti-chimer and anti-rBoNT/E-HCC antibodies) were able to recognize other proteins. Structural studies with circular dichroism showed that chimer proteins have slightly more secondary structures than rBoNT/E-HCC. **Conclusion:** The immunological results suggested that the above-mentioned identical region in rBoNT/E-HCC is more exposed. Circular dichroism, computational protein modeling and hydrophobicity predictions indicated a more exposed location for the identical region in rBoNT/E-HCC than the chimer protein, which is strongly in agreement with immunological results. *Iran. Biomed. J. 16 (4): 185-192, 2012*

Keywords: Botulinum neurotoxin type E (BoNT/E), Cross ELISA, circular dichroism, Computational modeling, recombinant vaccine-candidates

INTRODUCTION

Botulism is a dangerous neuroparalytic syndrome caused by blocking acetylcholine release at the neuromuscular junctions [1, 2]. Botulinum neurotoxins (BoNT), which cause botulism, are produced in seven different serotypes (A, B, C, D, E, F and G) by *Clostridium botulinum* strain [3]. Botulism syndrome is classified into three forms: the food-born, wound, and infant (intestinal) botulism [1, 3]. BoNT are initially produced as a stable complex of approximately 900 kDa and then divided into a 150-kDa neurotoxin and non-toxic components [1]. The 150-kDa neurotoxin consists of two polypeptide chains: a light chain (50 kDa) and a heavy chain (100 kDa), which are linked through a disulfide bond [4]. The light chain is organized as an N-terminal catalytic domain, while the heavy chain comprises an internal translocation domain and a C-terminal receptor binding domain.

The heavy chain, via receptor-mediated endocytosis, mediates translocation of light chain across the endosomal membrane into the cytosol. BoNT recognize nerve membranes by binding to two components: a group of membrane glycophospholipids called gangliosides and specific protein receptors such as synaptotagmin (for BoNT/D and G) or synaptic vesicle membrane protein, SV2 (for BoNT/A and E)

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[5, 6]. The light chain is a protease that cleaves target proteins in nerve cells such as synaptosomal-associated protein of 25-kDa and vesicle membrane protein synaptobrevin. Cleavage of these proteins causes the blockage of acetylcholine release and finally neuroparalysis [7, 8].

Vaccination against botulism by toxoids has some limitations, including the need for specific equipments which leads to high cost, the low yield of toxin production by *Clostridium botulinum* strain, the danger of *C. botulinum* handling, and the potential side effects and unexpected immunological reactions. To prevent botulism, researchers have been recently interested in using recombinant BoNT-based proteins as vaccine [9-11]. These types of vaccine have resolved many previous concerns related to use of toxoids. An example of these recombinant proteins is based on BoNT-binding domains with multivalent and monovalent antigenic properties [12]. Antibodies against these recombinant vaccines are proven to be effective in neutralizing BoNT effects [12]. The multivalent vaccines are more preferable than monovalent vaccines due to their ability to immunize against multiple neurotoxin serotypes.

Here, we study two of these binding domain-based recombinant proteins whose BoNT neutralizing ability has been previously reported. These proteins include a multivalent chimer protein (187 amino acid) which is composed of serotypes A, B and E binding subdomains [13] and a monovalent recombinant protein (259 amino acids) which contains 93 amino acid residues of C-terminal heavy chain of BoNT type E (rBoNT/E-HCC) [14]. Both of these proteins have an identical region (48 aa) that contains one of the most important BoNT/E epitopes (YLTHMRD sequence) [12]. The protein sequences and their homology have been depicted in Figure 1. In this study, the scale of antibody production against two above-mentioned recombinant proteins in rabbits was compared. Furthermore, we characterized some features of these vaccines as a criterion of multivalent and monovalent vaccine comparison by ELISA. Finally, we further confirmed the results of other studies using circular dichroism and molecular modeling [15].

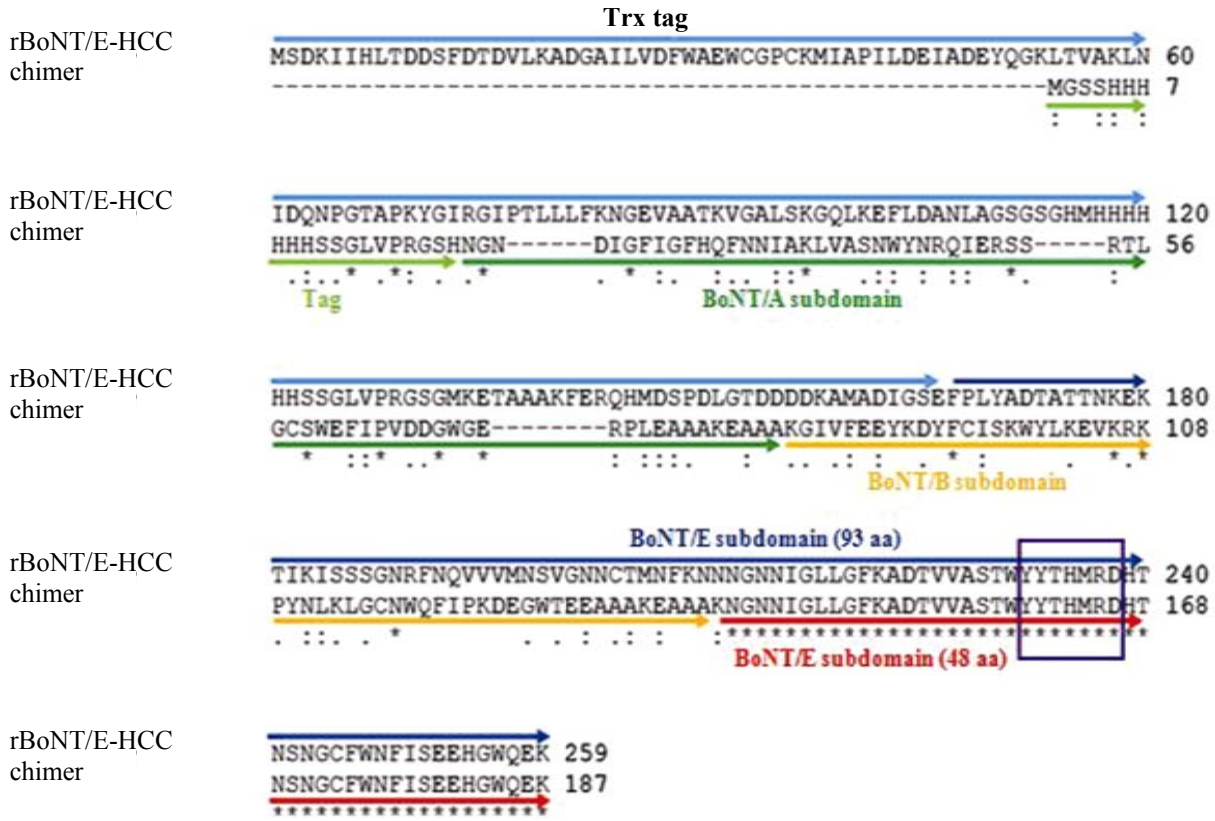

Fig. 1. Sequence alignment of recombinant C-terminal heavy chain of BoNT/E (rBoNT/E-HCC) and chimer protein. The amino acid sequences are numbered from the aminoterminal of proteins. Different parts of the proteins have been depicted with different colors. Chimer protein is composed of a tag of 58 aa from BoNT/A (BoNT/A subdomain), 61 aa from BoNT/B (BoNT/B subdomain), and 48 aa from BoNT/E (BoNT/E subdomain) from N- to C-terminal, respectively. rBoNT/E-HCC is composed of a tag, and 93-aa of BoNT/E from N- to C-terminal, respectively. The red arrow (48 aa) depicts a region of chimer protein, which is exactly similar to 48-aa sequence of rBoNT/E-HCC (the identical region). The purple box shows the active BoNT/E-epitope (YLTHMRD sequence) of the proteins. Trx tag shows a Trx•Tag™ thioredoxin protein in pET 32 [16]. For interpretation of the references to color in this text, the reader is referred to the web version of the article.

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MATERIALS AND METHODS

Bacteria, chemicals and media. All molecular biology grade chemicals and bacterial culture media were from Merck (Germany). Chemical agents for nickel nitrilotriacetic acid agarose (Ni-NTA) resin were from Qiagen (USA). LB powder was obtained from Difco (Sparkes, MD, USA). The pET-contained *E. coli* strains (pET32a encoding the sequence for rBoNT/E-HCC protein and pET28a, encoding the sequence for chimer protein were used to express recombinant proteins [13, 14].

Amino acid sequence alignments. Comparison of amino acid sequences of rBoNT/E-HCC and chimer protein (Fig. 1) was carried out using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) [17].

Recombinant protein expression and purification. The expression process was conducted as reported elsewhere [13, 14, 18]. After final step of protein expression process, the chimer protein was sufficiently pure, while the rBoNT/E-HCC solution was followed by further purification as mentioned below. The all separated protein fractions were run on 12% SDS-PAGE and stained with Coomassie blue. A 50% Ni-NTA bead suspension was used for purification of rBoNT/E-HCC protein [18]. Buffer E (100 mM NaH_2PO_4, 10 mM Tris-HCl and 8 M urea, pH 4.5) was applied to elute the expressed protein from Ni-NTA column. The purity of the sample was determined by SDS-PAGE analysis and a single protein band (31 kDa) was obtained.

Antigenicity testing. In order to compare the recombinant protein antigenicity, purified recombinant proteins were mixed with an equal volume of complete Freund's adjuvant for initial injection and incomplete Freund's adjuvant for subsequent injections into 3 rabbits intradermally. Each rabbit received 100 µg of antigen at weeks 0, 2 and 4. Two rabbits were used as controls receiving only adjuvant. A week after second and third injections, the animals were bled and sera were restored for ELISA experiments. ELISA plates were coated with an optimal concentration of purified proteins (3.5 µg ml^{-1} per well) in a coating buffer (15 mM Na_2CO_3 and 36 mM $NaHCO_3$, pH 9.8) and allowed to adhere at 4°C overnight. Also, one row was incubated with coating buffer alone (no-antigen) as control. Plates were washed four times with 400 µl PBST and blocked with 100 µl per well of skim milk (50 mg ml^{-1}) at 37°C for 45 min. After washing, a serial two-fold dilutions in PBST, starting at 1:200, of rabbit serum samples were then added (100 ml per well) and plates were incubated at 37°C for 30 min. Following a washing step, plates were incubated at 37°C for 30 min with anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (1:2,000; 100 µl per well) in PBST. Plates were washed four times with 400 µl PBST and then incubated with 100 µl per well of *ortho*-phenylenediamine (Sigma, USA) and H_2O_2 as substrate. The reaction was terminated by addition of 2.5 M H_2SO_4 (100 µl/well) and the absorbance was read at 490 nm using an ELISA plate reader (Anthos2020, Eugendorf, Austria).

Antibody purification. Protein-G-Sepharose columns (Sigma, USA) were used to purify IgG from sera. After washing the columns with 100 mM and then 10 mM Tris (pH 7.8), the sera were mixed 1:10 with Tris (1 M) and applied to the columns. The columns were washed again as indicated above and purified IgG was eluted using 100 mM glycine, pH 3.0.

Circular dichroism spectropolarimetry. In order to obtain information about the secondary structure of the recombinant proteins, circular dichroic spectra were gathered in far-UV regions (195-260 nm) using a JASCO model J-810 spectropolarimeter at 25°C. The cell volume was approximately 0.5 ml with a path length of 0.1 mm. The recombinant proteins were at a concentration of 0.25 mg ml^{-1} in 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0. The secondary structures of proteins were determined using CDNN program, version 2.1.0.223 (obtained from Institute of Biophysics and Biochemistry of Tehran University, Dr. Moosavi-Movahedi Laboratory).

Computational molecular modeling of the rBoNT/E-HCC and chimer protein. The tertiary structures of rBoNT/E-HCC and chimer protein are unknown. Here, we obtained *de novo* protein structure predictions at the Robetta [19, 20] and Lomets [21] servers. The evaluation of the quality of the models was performed with Qmean server [22]. The molecular dynamic simulations were carried out using HyperChem Professional version 8.0 Molecular Modeling System [23]. Charmm27 force field [24] was used for the simulations according to the procedure described by Fendri *et al.* [25]. The energy minimization of these proteins was carried out under implicit solvent conditions using the conformational analysis programs. The lowest energy conformations were then solvated with TIP3P water explicitly, and finally the overall system was energy minimized using the Polak-Ribiere conjugate gradient method until the convergence of the gradient (0.01 kJ mol^{-1}) [23]. PyMol version 0.99 beta 06 (http://www.pymol.org), UCSF Chimera and MSMS were used to produce molecular graphics images and other representations [26, 27].

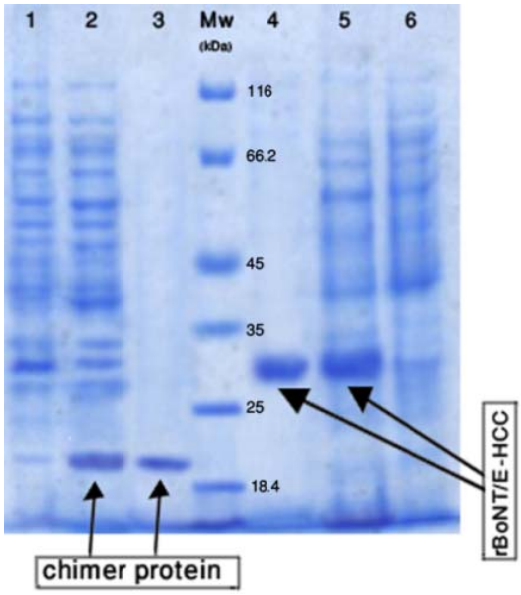

Fig. 2. SDS-PAGE analysis of recombinant protein expression and purification. The separated protein fractions were run on 12% SDS-PAGE gel and stained with Coomassie blue. Lanes 1 and 2, cell lysate of *E. coli* BL21 (DE3) containing pET28a-chimer gene before and after induction with isopropyl-1-thio-b-D galactopyranoside (IPTG), respectively; Lanes 5 and 6, cell lysate of *E. coli* BL21 (DE3) containing pET32a-rBoNT/E-HCC (recombinant C-terminal heavy chain of BoNT/E) gene after and before induction with IPTG, respectively. The expressed recombinant proteins were observed at approximately 20 and 31 kDa; Lane 3, purified chimer protein obtained after final ultracentrifugation in buffer II (200 mM NaCl, 50 mM Tris, 8 M Urea and 2.5 M glycerol) [13]; Lane 4, purified rBoNT/E-HCC by Ni-NTA agarose affinity column after adding wash buffer E (100 mM NaH_2PO_4, 10 mM tris-base, 8 M urea, pH 4.5) [18]. Lane Mw shows protein molecular weight marker.

RESULTS AND DISCUSSION

Expression and purification of recombinant proteins. The recombinant expression vectors (encoding the desired recombinant proteins) with a His_6 tag were prepared in the T7-promoter-based pET vectors (pET32a (+) for rBoNT/E-HCC and pET28a (+) for chimer protein) in *E. coli* BL21 (DE3) cells as previously described [13, 14]. The cells were grown in LB broth and expression was induced using isopropyl-1-thio-b-D galactopyranoside. Each culture sample was evaluated for protein expression by SDS-PAGE (Fig. 2, Lanes 1, 2, 5 and 6). The rBoNT/E-HCC in bacteria supernatant was allowed to selectively bind to Ni-NTA agarose gel through its His_6 tag and eluted using the elution buffers (100 mM NaH_2PO_4, 10 mM Tris-HCl and 8 M urea,). The purified recombinant protein was analyzed by SDS-PAGE showing a 31-kDa band corresponding to the rBoNT/E-HCC (Fig. 2, Lane 4). The purified recombinant chimer protein also showed a single band (~20 kDa) on SDS-PAGE (Fig. 2, Lane 3).

Characterization of antibody responses to recombinant proteins. ELISA was performed to determine antibody titers of sera from rabbits vaccinated against the recombinant proteins. Two weeks after last vaccination, blood samples were taken and used for ELISA. Produced antibody titers were observed in rabbits immunized by rBoNT/E-HCC or chimer protein (Fig. 3).

Recombinant protein and purified IgG interactions. The chimer protein and anti-chimer protein IgG, and rBoNT/E-HCC and anti-rBoNT/E-HCC IgG interactions were evaluated by ELISA. Approximately 8 ng of anti-rBoNT/E-HCC IgG and anti-chimer protein IgG were able to react with their respective antigen (Fig. 4). Since both proteins had an identical region, in order to test cross reactivity between rBoNT/E-HCC and anti-chimer protein serum/IgG, and chimer protein and anti-rBoNT/E-HCC serum/IgG, ELISA was applied. Once rBoNT/E-HCC was coated on ELISA plate wells, the anti-chimer protein serum and purified IgG dilutions were added separately (Fig. 5), and alternatively chimer protein was coated and anti-rBoNT/E-HCC serum and purified IgG were added separately (Fig. 5). These studies showed that antibodies to recombinant proteins were generated and recognized their respective antigen. However, the antibodies affinity and titers were significantly higher in rBoNT/E-HCC compared to chimer protein. It may refer to the recombinant protein structural

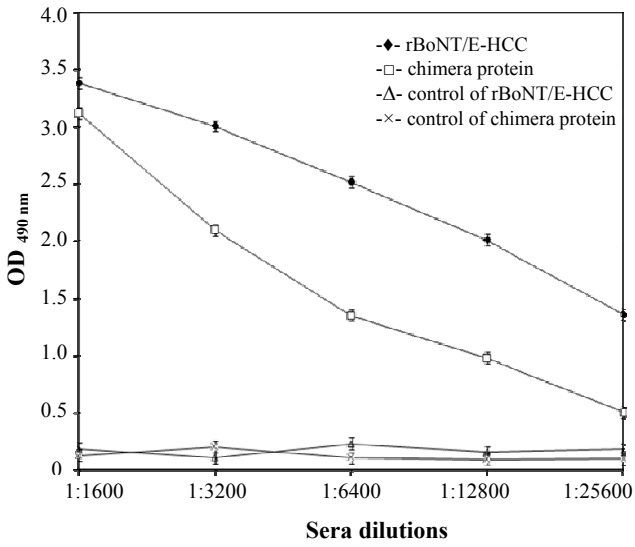

Fig. 3. ELISA results of rabbit serum antibody titration against rBoNT/E-HCC and chimer proteins. Both anti-rBoNT/E-HCC and anti-chimer protein sera from rabbits bind to their antigens up to 1:25,600; however, anti-rBoNT/E-HCC (recombinant C-terminal heavy chain of BoNT/E) serum had higher affinity. Serum from non-immunized rabbit did not react with either rBoNT/E-HCC or chimer protein. The error bars present high reproducibility of double repeats of the experiment.

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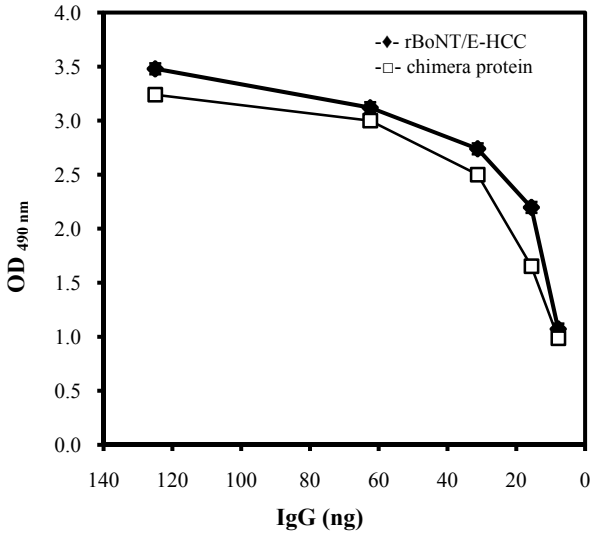

Fig. 4. Comparison of ELISA results of anti-rBoNT/E-HCC (recombinant C-terminal heavy chain of BoNT/E) and anti-chimer protein IgG reactions with their antigens (rBoNT/E-HCC and chimer protein, respectively). Approximately 8 ng of anti-rBoNT/E-HCC and anti-chimer protein IgG could signify and bind to their antigens. In comparison with anti-chimer protein, IgG and chimer protein reaction, anti-rBoNT/E-HCC IgG reacted with its target protein (rBoNT/E-HCC) with higher affinity. The error bars present high reproducibility of double repeats of the experiment.

features, which lead to different identical region (i.e. BoNT/E-epitope containing region) locations.

Circular dichroism. ELISA results showed that the different structural features of the recombinant proteins may lead to more exposure of the identical region in rBoNT/E-HCC. To verify this hypothesis and also gain insight into structural properties of the recombinant proteins, circular dichroism was applied. Circular dichroism spectra of both rBoNT/E-HCC and chimer protein were taken at 25°C to determine their secondary structures. The amount of secondary structures was higher in chimer protein in comparison with rBoNT/E-HCC (Fig. 6). Circular dichroism spectra analysis using CDNN program, version 2.1.0.223, confirmed these results (Table 1). These results support our hypothesis and indicate in rBoNT/E-HCC, where the amount of secondary structures is less, the protein tends to be less ordered and may affect BoNT-epitope location to be more exposed.

Theoretical studies. After achieving some insights into the proteins compactness by circular dichroism, computational studies were used to characterize and evaluate the mentioned hypothesis. The sequences of rBoNT/E-HCC and chimer protein were subjected to *de novo* tertiary structure predictions for conformational epitope determinations. An ensemble of tertiary structures for each protein was obtained using the modeling programs Robetta [19, 20] and Lomets [21]. These programs were used to generate *de novo* protein structure prediction models. A set of 15 three-dimensional models was generated for each protein (10 from Lomets and 5 from Robetta). To

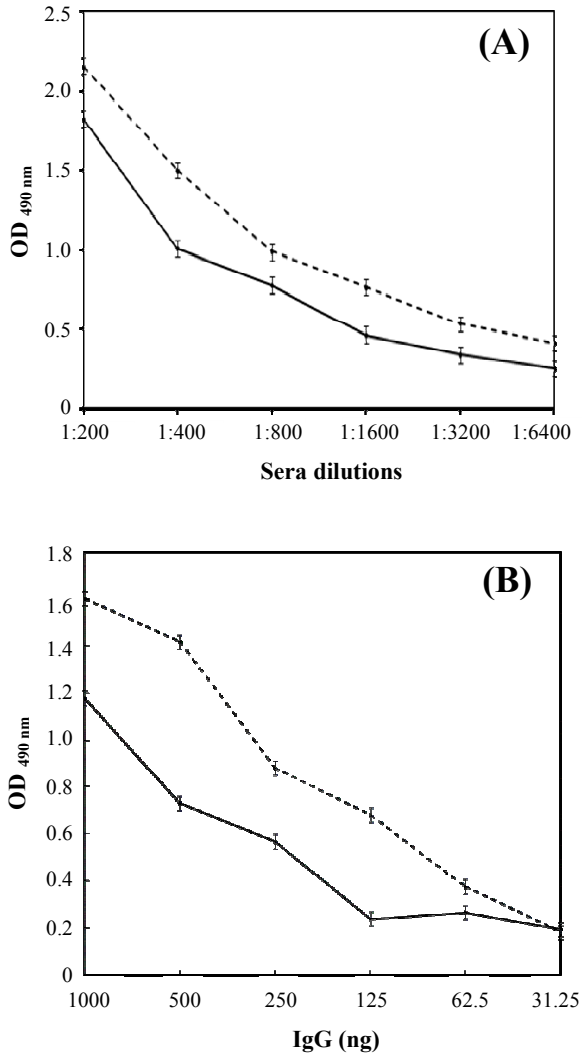

Fig. 5. ELISA results of cross reaction between rBoNT/E-HCC and anti-chimer protein serum/IgG, and chimer protein and anti-rBoNT/E-HCC (recombinant C-terminal heavy chain of BoNT/E) serum/IgG. Assuming OD 0.5 as a reliable answer: **(A)** both anti-rBoNT/E-HCC and anti-chimer protein sera could bind to the other proteins up to appropriately 1:1,600 dilution. However, anti-rBoNT/E-HCC interacts more strongly. **(B)** Approximately 125 ng of both anti-rBoNT/E-HCC and anti-chimer protein IgG could interact with the other recombinant antigens. However, anti-chimer IgG react with rBoNT/E-HCC more efficiently that may be due to the more exposed location of epitopes in rBoNT/E-HCC. The dash line depicts the interaction of rBoNT/E-HCC with chimer serum (panel A) or IgG (panel B); the solid lines depict the interaction of chimer protein with rBoNT/E-HCC serum (panel A) or IgG (panel B). The error bars represent high reproducibility of double repeats of the experiments.

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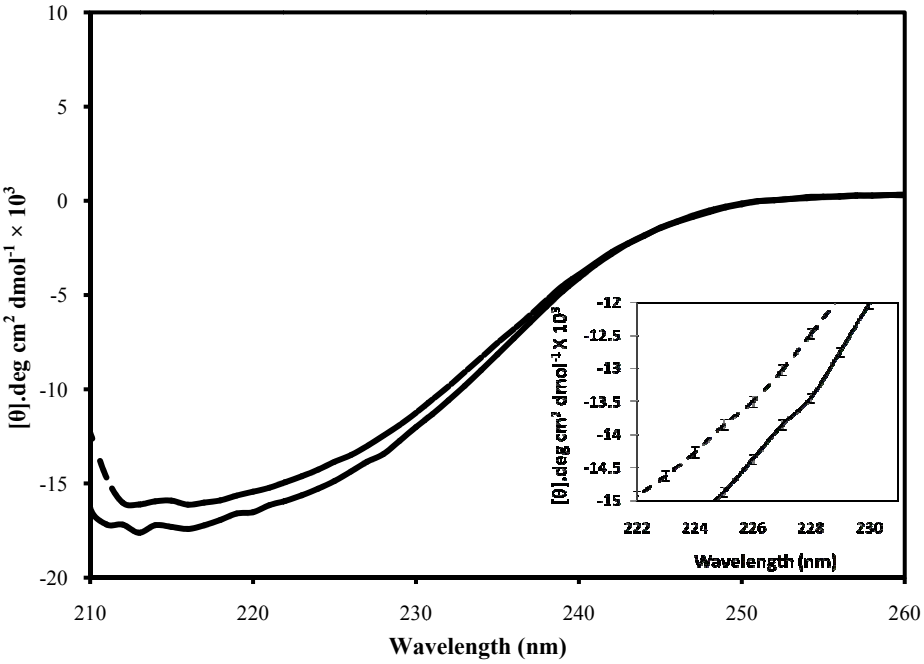

Fig. 6. Circular dichroism spectrum of recombinant proteins. The CD spectrum was recorded as described in materials and methods. Chimer protein has more secondary structures. The dash and solid lines show the circular dichroism pattern of rBoNT/E/HCC and chimer protein, respectively. Error bars of spectra for duplicate repeating experiments and high reproducibility are shown in the inset.

select the best three-dimensional model, the Qmean server [22] was used for model quality estimation. Based on the global score of the whole model, the highest predicted model reliability was measured from 0 to 1 (1 as the highest quality estimation). For both proteins, one of the models generated by the Robetta server yielded the highest value of 0.781 and 0.666 for rBoNT/E-HCC and chimer protein, respectively. These models were used thereafter for mapping conformational epitopes. PyMol and UCSF Chimer were used to produce molecular graphic images. The resulting models have been shown in Figure 7. Panel A shows a molecular surface representation of each protein, and panel B shows the hydrophobicity surface of the corresponding molecules in the same orientation of panel A. Figure 7 indicates that the identical region location in chimer protein is in a more hydrophobic region which leads to burying the epitope into the protein. Altogether, Figure 7 shows that identical region location in rBoNT/E-HCC seems to be more exposed. These results are in full agreement with those of immunological and circular dichroism studies.

Previous works by others have focused on design of recombinant vaccines against a single BoNT serotype (monovalent) or against a group of them (multivalent) [10]. Here, we focused on two recombinant vaccines against BoNT whose abilities to neutralize BoNT were previously reported [13, 14]: a multivalent chimer protein (187 amino acid residues) which is composed of types A, B and E binding subdomains, and a monovalent recombinant protein (259 amino acid) which contains 93 amino acid residues of rBoNT/E-HCC. The monovalent vaccine is able to neutralize BoNT/E more efficiently [13, 14]. Since both recombinant proteins have a 48 aa-identical region (that contains one of the most important BoNT/E epitopes [YLTHMRD sequence, Fig. 1]), we designed some experiments to characterize their antibody interaction with the opposite protein (i.e. rBoNT/E-HCC with anti-chimer protein serum/IgG, and chimer protein with anti- rBoNT/E-HCC serum/IgG). We also determined their structural as well as other immunological feature differences.

Immunological studies showed that both proteins were able to evoke high antibody titers in rabbits (as an animal model); however, the antibody titers against rBoNT/E-HCC were higher. Cross ELISA experiments confirmed that antibodies against chimer protein recognize rBoNT/E-HCC more efficiently, though both antibodies were able to recognize the other recombinant antigen. These results suggest that the epitope sequence against the identical region (i.e.

Table 1. Circular dichroism spectra analysis by CDNN program version 2.1.0.223.

2nd structure	rBoNT/E-HCC (%)	Chimer (%)
Helix	38.51	39.80
strand and turn	29.35	29.56
Rndm. coil	32.14	30.64
Total sum	100.00	100.00

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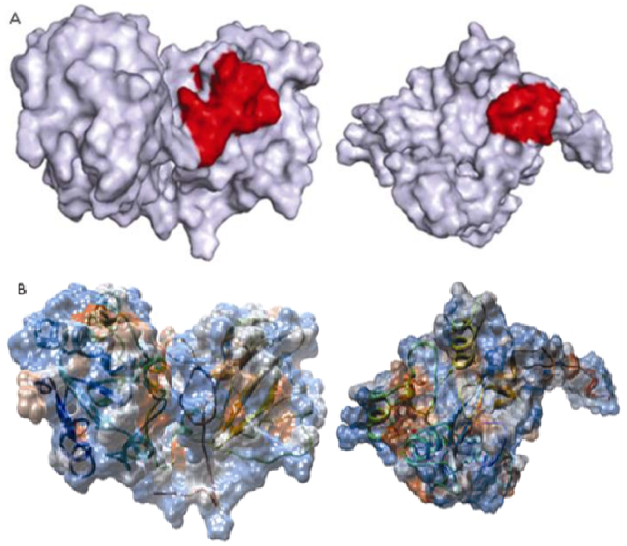

Fig. 7. Three-dimensional molecular models of recombinant rBoNT/E-HCC (recombinant C-terminal heavy chain of BoNT/E) and chimer protein. Panel **A** shows the surface representation of rBoNT/E-HCC (left) and chimer protein (right), the structure visualization by PyMol version 0.99 beta06. Red parts show the BoNT/E-epitope location in proteins. Panel **B** depicts the hydrophobicity surfaces of each protein in the same orientation of panel A. The hydrophobicity surface shows the amino acid hydrophobicity with colors ranging from dodger blue for the most hydrophilic to white at 0.0 to orange red for the most hydrophobic. The images were made with UCSF Chimer [27] and the surface was calculated with the MSMS package [26]. For interpretation of the references to color in this text, the reader is referred to the web version of the article.

BoNT/E-epitope, YLTHMRD) in rBoNT/E-HCC is more exposed in comparison with the chimer protein and it may refer to the differences in their structure.

Therefore, structural studies were used to confirm this hypothesis. Structural studies with circular dichroism showed that chimer protein have slightly more secondary structures than rBoNT/E-HCC. Differences of circular dichroism pattern between these two recombinant proteins, which refer to their structural differences, may affect the identical region (BoNT/E-epitope) location. In rBoNT/E-HCC, where the amount of secondary structures is less, the protein tends to be less ordered and may affect on the identical region (BoNT-epitope) location to be more exposed. Protein modeling and calculations of hydrophobicity showed a more exposed location for the identical region (BoNT/E-epitope) in rBoNT/E-HCC than the chimer protein. These results are in full agreement with the immunological and circular dichroism studies. Furthermore, the lower antibody titers of chimer protein (lower efficiency of the multivalent vaccine), and lower affinity between chimer protein and anti-rBoNT/E-HCC antibodies were influenced by the identical region (BoNT/E-epitope) positions in the two proteins. Altogether, the results suggest that due to the epitope location, rBoNT/E-HCC seems to be a better candidate for use as vaccine against BoNT/E than chimer protein, though more studies should be performed in this area.

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