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The Effect of pH on Recombinant C-terminal Domain of Botulinum Neurotoxin Type E (rBoNT/E-HCC)

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A B S T R A C T

Recombinant proteins are tending to be the most favorable vaccine-candidates against botulism. Recombinant Carboxy-terminal of botulinum neurotoxin serotype E (rBoNT/E-HCC) has been introduced as an efficient vaccine against botulism type E. In this report, we made an effort to investigate the effect of different pH on protein structure to assess if rBoNT/E-HCC could be used as a vaccine for oral administration. Initially, rBoNT/E-HCC was expressed and purified. Structural changes of rBoNT/E-HCC at several pH conditions were studied by various techniques including circular dichroism (CD), fluorescence, aggregation and UV-Vis spectroscopy. The results showed the more compact and more stable structure for rBoNT/E-HCC at acidic pH, and loosely folded structure at alkaline pH. Our finding as the first step of rBoNT/E-HCC evaluation, hopefully introduce it as a suitable vaccine candidate for oral administration.

Key words: Botulinum Neurotoxin Type E, pH, Fluorescence, Circular Dichroism, Aggregation.

INTRODUCTION

Botulinum neurotoxin is the most potent bacterial protein toxin that is produced by *Clostridium botulinum* [1, 2]. Up to now, seven serotypes of this neurotoxin have been identified and classified using A to G letters [3]. There are several reports indicating that the most important serotypes involving in human botulism are serotype A, B, D and E [4, 5]. The neurotoxins (BoNTs) are synthesized as a single chain precursors with molecular weight of about 150 KDa. The polypeptide chain of neurotoxin is subsequently cleaved by bacterial proteolytic enzymes [6, 7]. This produces a ~50 KDa light chain (LC) fragment from the protein N-terminus and a ~100 KDa heavy chain (HC) fragment from C-terminus [1, 8, 9]. A single disulfide bridge connects these two fragments. HC fragment comprises

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two functional domains with different functions; an internal domain which is responsible for toxin translocation to host cells and a C-terminal domain responsible for toxin binding to its receptor. LC fragment is zinc dependent catalytic domain of neurotoxins [10]. Structure flexibility by assistance of several residues of light chain interacted with heavy chain allows LC to be translocated through the membrane channel [11, 12]. LC fragment in host cells hydrolyzes cellular SNARE (soluble N-ethylmaleimide–sensitive fusion protein attachment protein receptor) proteins such as vesicle associated membrane protein/synaptobrevin, synaptosomal-associated protein or syntaxin at neuromuscular junctions. Cleavages of SNARE proteins which are important in acetylcholine releasing, eventually leads to flaccid paralysis [13-16].

Nowadays, two kinds of vaccines are available for prevention of botulism toxicity, a multivalent vaccine for types A, B, C, D and E; and a monovalent vaccine for each types of A to G [17, 18]. Unfortunately, these vaccines are of limited accessibility all around the world and the risk of toxin reactivation of these vaccines is remained conflicting. Because of these limitations, recombinant vaccines are preferred as new vaccines [19]. The most common strategy for designing of botulism recombinant vaccine is based on the fragments of binding domain of neurotoxin serotypes which contains two dominant epitopes [20, 21]. One of the proposed recombinant vaccines which its proficiency as vaccine has been reported is an epitope-including fragment (93 residues) from C-terminus of botulinum neurotoxin type E ("recombinant botulinum neurotoxin type E heavy chain C terminal" or briefly "rBoNT/E-HCC") [22, 23].

Since producing a vaccine with least side effects is strongly desirable, vaccine producing industries attempt to develop oral vaccines which have several benefits such as mucosal immunity provoking, patient comfortableness and no need of highly qualified personnel for vaccination [24]. Although mucosal immunity of some BoNTs binding domain-based vaccine candidates was evaluated [19], there is no report regarding to mucosal immunity evaluation of rBoNT/E-HCC.

In our previous study we showed that rBoNT/E-HCC is able to provoke high titers of antibody in animal models when it is administrated intravenous [23]. Here, in aim of rBoNT/E-HCC evaluation as an oral vaccine, the effect of pH as the main factor in developing of oral vaccines was studied using different methods.

MATERIALS AND METHODS

Materials: All molecular biology grades chemicals and media for culturing the bacteria were obtained from Merck (Germany). Chemical agents for nickelnitrilotriacetic acid (Ni-NTA) resin were purchased from Qiagen (USA). LB (Lauria Bertani) powder was purchased from Difco (Sparks, MD, USA). The pET-contained *E.coli* strain was obtained from Agheli-Mansour, *et al*. [22].

rBoNT/E-HCC expression: The expression was performed as reported elsewhere [25]. Briefly the bacteria were cultured in LB broth, induced by 1 mM isopropyl-1-thio- -D galactopyranoside (IPTG), and finally centrifuged ($3200 \times g$, 25°C, 6 min) to collect the

cells. 20 µl of cell lysate was loaded in 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide) gel.

rBoNT/E-HCC Purification and Dialysis: As previously reported, purification of rBoNT/E-HCC as a polyhistidine tagged protein extract was done using a 50% Ni-NTA resin (NTA) with several washing buffers (C, D, E and MES buffers) [25]. The purified proteins electrophoresed on a 12% SDS-PAGE to confirm the purification. After protein purification, the rBoNT/E-HCC solutions were dialyzed in order to remove urea (8 M). For this purpose we used a dialysis bag that its cut-off-number was 12-KDa. After dialyzing, the protein samples were restored at -20°C for following structural studies.

Fluorescence spectroscopy: In order to carry out intrinsic fluorescence experiments, a spectrofluorescence (Cary Eclipse Varian, Australia) which has a bath to control temperature was applied. 280 nm was applied as the excitation wavelength. The protein samples emission spectra were obtained at 300-500 nm in 10 nm bandwidth. The protein samples were at concentration of 0.1 mg/ml in buffer B (100 mM NaH_2PO_4, 10 mM Tris–HCl) at four pH values (2, 5, 7.4 and 9).

Circular dichroism (CD) spectropolarimetry: Circular dichroic spectra were obtained in far-UV regions (195-260nm) on a model J-810 Jasco spectropolarimeter at 25°C. The cuvette volume was approximately 0.5 ml, 1 millimeter path length. Concentration of the protein was 0.25 milligram per milliliter in buffer B (100 mM NaH_2PO_4, 10 mM Tris–HCl) and four different pH values (2, 5, 7.4 and 9). Protein secondary structures were taken using version 2.1.0.223 of CDNN program.

Aggregation: The aggregation of rBoNT/E-HCC (with concentration of 0.2 mg/ml in buffer B and four pH of 2, 5, 7.4 and 9) were studied by recording absorbance at 360 nm in a spectrophotometer (Cary-100 Bio VARIAN) by using of a quartz cuvette (with path length of 10 mm) [26]. Aggregation was studied by adding 50 mM dithiothreitol (DTT) to each sample to reduce disulphide bonds, as was described by Rudolph *et al.* [27]. Aggregation was continued for 180 min at 50°C.

RESULTS

rBoNT/E-HCC sequence: Figure 1 depicts rBoNT/E-HCC primary structure. The rBoNT/E-HCC contains the total of seventeen ionizable residues, six with negative charges (3 Asp and 3 Glu), eight with positive (2 Arg and 6 Lys), and three His which carry 3 positive charges at pH under 6. Considering these residues the net charge of protein at moderate pH is predominantly positive.
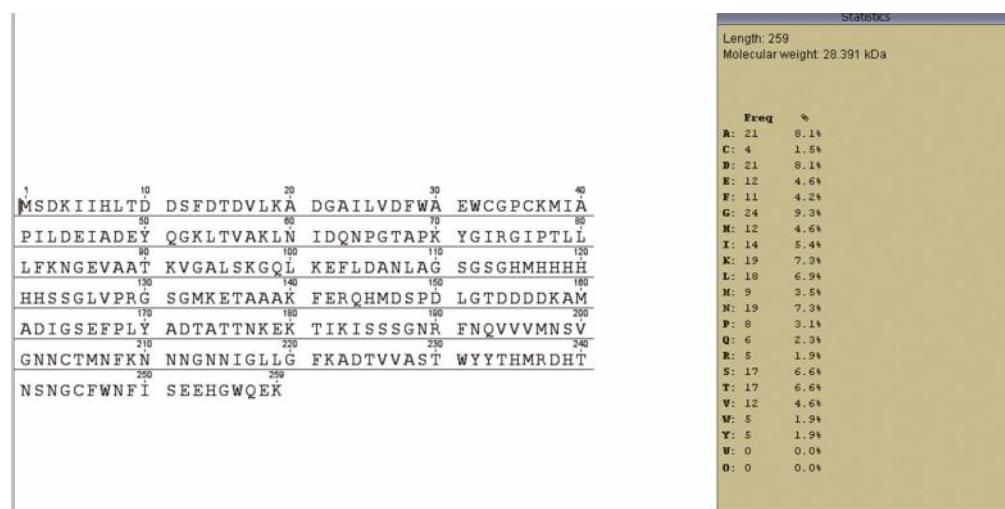
Figure 1: Complete sequence of rBoNT/E-HCC which is illustrated using Geneious R6 application.

Expression and purification of rBoNT/E-HCC: The rBoNT/E-HCC gene with a 6-His tag was utilized to express the recombinant protein. Each culture samples was tested for the presence of proteins using SDS polyacrylamide gel (12%) electrophoresis. The pET32a (+) system fused a ~ 18 KDa Trx family tag, containing 6-His tag to the gene [28]. Thus the final protein (Trx tag fused to 93 amino acid from C-terminal of botulinum neurotoxin type E), rBoNT/E-HCC, is expected to have a molecular weight of ~ 29 KDa. The rBoNT/E-HCC gene was highly expressed and the expression band was observed about 29 KDa (Figure 2-a). Purifying of rBoNT/E-HCC by Ni-NTA agarose column concluded a single band (~29 KDa), showing a highly purified protein, (Fig. 2-b). Our previous immunological study [23], introduced rBoNT/E-HCC (including it's His tag) as a vaccine candidate, so here we did not omit the His-tag to be able to link our results with the previous immunological result.

Fluorescence measurements: Fluorescence spectroscopy was used in structural study of rBoNT/E-HCC at different conditions. Figure 3 shows maximum emission of rBoNT/E-HCC at pH 2, 5, 7.4 and 9, at 349 nm with excitation wavelength of 280 nm. These emission patterns are characteristic of tryptophan residues (5 Trp of rBoNT/E-HCC) [29]. As it is depicted in figure 3, increasing the pH from 2 to 9 results in increasing of emission intensity directly. Considering the positive net charge of protein, the interpretation of the emission intensification seems to be easy. At pH 2 the protein takes a more compacted structure because of repulsive interactions between protein's positive net charges and H^+ ions of buffer. The more compacted structure of rBoNT/E-HCC at acidic pH probably leads to quenching effects of other residues such as Arg or leads to more burial (less exposure) of Trp, result in preventing of attenuated emission producing [30]. Increasing the pH decreases the repulsive interactions between the protein and its environment, allowing rBoNT/E-HCC to take a more relaxed tertiary structure. These structural changes, increases the quantum of Trp exposure and ultimately causes hyperchromic effect.
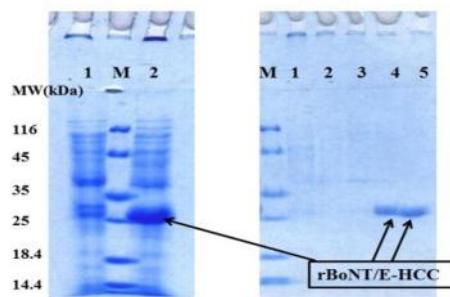
Figure 2: SDS-PAGE analysis of rBoNT/E-HCC expression in *E. coli* BL21 DE3. Columns 1 and 2 demonstrate cell lysate of *E. coli* BL21 DE3 containing pET32a before and after induction with IPTG respectively. Column M shows protein molecular weight marker. The expressed band was observed about 29-KDa. b) Purification of rBoNT/E-HCC on a Ni–NTA agarose affinity column. The separated protein fractions were run on 12% SDS–PAGE gel and stained with Coomassie blue stain. Column M, protein molecular weight marker; column 1, supernatant/soluble fraction after lysis of cells; column 2, wash buffer C (pH 6.9); column 3, wash buffer D (pH 5.9); column 4, wash buffer E (pH 4.5); column 5, 2-N Morpholino ethane sulfonic acid (MES) eluent. All C, D and E buffers have the same content (NaH$_2$PO$_4$ (100 mM), tris-base (10mM), Urea (8M)) whereas their pH is different. Purified rBoNT/E-HCC was obtained after adding E buffer and MES eluent.
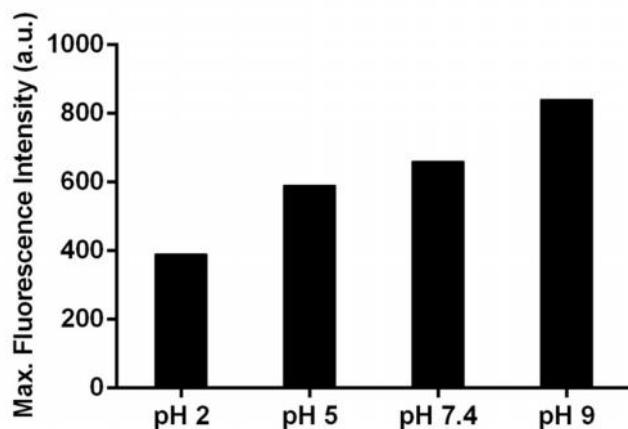
Figure 3: Fluorescence intensity of rBoNT/E-HCC solution in buffer B at pH 2, 5, 7.4 and 9 at 37°C. Increasing the pH from 2 to 9 amplify the emission intensity in a direct relation.

Circular dichroism and protein secondary structure: In order to investigate secondary structure alterations induced by different pH in rBoNT/E-HCC, circular dichroism spectra of the protein were obtained at pH 2, 5, 7.4 and 9. Figure 4 shows CD spectra of rBoNT/E-HCC and table 1 summarizes the corresponding secondary structures

obtained by spectral analysis using CDNN software, version 2.1.0.223. The results show that the major constituent of rBoNT/E-HCC secondary structure (>40%) is alpha helix. About 21% of residues take a random structure, which seem essential for protein flexibility in its native conformation. At pH 2 regular structures including alpha helix, beta structure (parallel and anti-parallel) and beta turn comprise 80 percent of protein residues. Increasing of pH led to decreasing of the regular structures. The random coil percent of rBoNT/E-HCC at pH 2 (21.31%) was changed to 26.27% and 28.08%, at pH 5 and 7.4 respectively, while the main regular structure, alpha helix, was changed from 56.27% at pH 2 to 46.95% and 43.74% at pH of 5 and 7.4 accordingly.
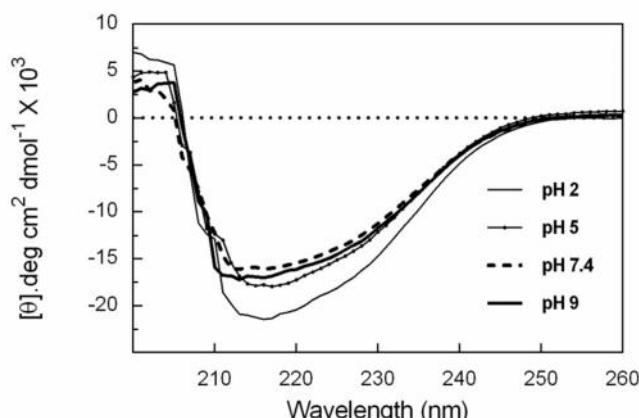
Figure 4: The far-UV CD spectra of the rBoNT/E-HCC at concentration of 0.25 mg/ml in buffer B (100 mM NaH_2PO_4, 10 mM Tris-HCl) at pH 2, 5, 7.4 and 9.

Table 1: The result of secondary structure analysis of rBoNT/E-HCC spectra (which are depicted in figure 4) by CDNN program, version 2.1.0.223.

pH	Percent of Secondary Structure				
	Helix	Beta-Sheet	Random Coil	Beta-Turn	Total
2	56.27	8.99	21.31	13.43	100
5	46.95	11.91	26.27	14.87	100
7.4	43.74	12.92	28.08	15.26	100
9	45.98	12.21	26.86	14.95	100

The pattern of secondary structures alterations from pH 7.4 to pH 9 seems to be unexpected; alpha helix amount was increased from 43.74% to 45.98% and random coil amount was decreased from 28.08% to 26.86%. If we assume that pH around 7.4 is isoelectric pH for rBoNT/E-HCC protein, the latest pattern may be interpreted. In such condition, it could be acceptable to expect that increasing pH from 7.4 to 9 decreases random coil structures and increases alpha helixes, although more studies should be done in this case. Furthermore the main difference is seen for pH 2 in which alpha helix

composes the major portion of the protein, and the slight differences between pH 5, 7.4 and 9 CD-pattern could be relinquished.

rBoNT/E-HCC Aggregation*:* thermal aggregation study of protein at 360 nm is a useful method to show the resistance of proteins to thermal denaturation. A thermal aggregation curve is composed of three phases, a lag phase, a logarithmic phase and a plateau phase [31, 32]. A large lag phase and a slow slope logarithmic phase indicate a more stable protein [32]. In order to simplify the aggregation experiment and prevent disulphide bond formation during experiment, which interferes with mechanisms involving in aggregation, we added 50 mM concentration of DTT to prevent disulphide bond formation. As it evident from Figure 5, the maximum rate of aggregation is seen in pH 9 and the lower rate for pH 2 which is in full agreement with this hypothesis that rBoNT/E-HCC has the more compacted and more stable structures at pH 2 and less stable structure at pH 9 [see ref. 30].
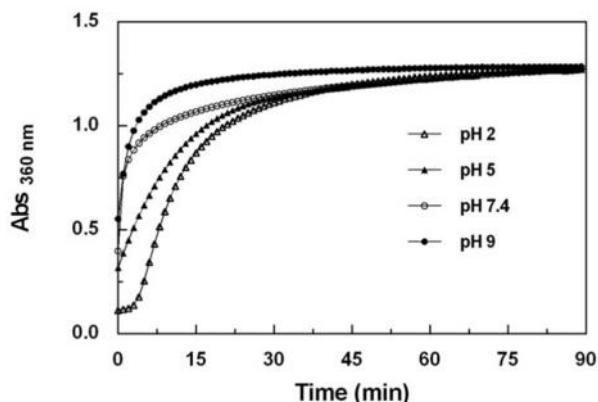
Figure 5: Thermal aggregation of rBoNT/E-HCC in the presence of 50 mM dithiothreitol for each sample. The more compacted and more stable structure of protein at pH 2 becomes more stable against thermal aggregation. Protein aggregation at pH 5, 7 and 9 increases with decrease in their stability accordingly.

DISCUSSION

In the present study different methods were used and their results were described according to the mechanism and the forces involved in each experiment. Fluorescence (Fig. 3) showed that increasing in pH from 2 to 9 expands protein from its compacted structure to slightly extended structure. CD studies showed that the protein tends to have more secondary structure at pH 2. Aggregation experiment (Fig. 5) indicated that rBoNT/E-HCC at 50°C aggregates in lower rate at pH 2. Altogether the results introduce a more compact and more stable form of rBoNT/E-HCC at acidic pH. It is noteworthy that observed structural difference on studied pH is not very extreme, so it is hard to judge

about the effect of these changes on the protein immunogenicity and more immunological studies are needed.

Although it seems that most proteins would be unfolded or partially folded at low pH, there may be an exception for Botulinum-based proteins, so that in the most relevant study to our finding, Bedu-addo *et al.* also indicated that optimal stability of rBoNTB(Hc) is seen at low pH [33].

Considering our results we can make a general conclusion as follow: i: exposure of rBoNT/E-HCC to different pH of 2, 5, 7.4 and 9 did not extremely change its secondary or tertiary structure (maximum change is about 6%). ii: It is thought that aggregation of proteins is undesirable in therapeutics use of protein [34]. Our results showed that rBoNT/E-HCC aggregation at physiological temperature of 37 ˚C is neglectable and in order to induce aggregation, 50°C is needed. iii) It seems that rBoNT/E-HCC is able to endure the harsh condition of gastrointestinal tract condition. It is stable at pH 2 (that is assumed as pH of stomach) and it also did not change extremely at alkaline pH that may consider as pH of other parts of gastrointestinal tract (intestine etc.).

Our finding as the first step of rBoNT/E-HCC structure evaluation, hopefully introduce it as a suitable vaccine candidate for oral administration, however we advise further studies on it in both biophysical and immunological point of view.

Acknowledgments

Conflict of Interest: Authors have no financial or any non-financial competing interests.

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