

# Immunogenic and protective potentials of recombinant receptor binding domain and a C-terminal fragment of *Clostridium botulinum* neurotoxin type E

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## Abstract

*Clostridium Botulinum* Type E neurotoxin heavy chain consists of two domains: the translocation domain as the N-terminal half and the binding domain as the C-terminal half (Hc). One effective way to neutralize botulinum neurotoxin is to inhibit binding of this toxin to neuromuscular synapses with antibodies against binding domain. Two synthetic genes, coding for Hc (the full length binding domain) and the c-terminal quarter of binding domain (HcQ), were cloned in pET-28a vector and over-expressed in *E. coli* BL21 (DE3) cells. These recombinant proteins were purified by affinity Ni-NTA column (under native condition). Mice were vaccinated with 2 µg of purified proteins, respectively; at step one with complete adjuvant, steps two and three with incomplete adjuvant and step four only with phosphate buffered saline (PBS). Enzyme-linked immunosorbent assay (ELISA) has been performed with mice serum samples 14 days following their third and final vaccination. Binding activity of the purified proteins to ganglioside and synaptotagmin II was analyzed by ELISA. The results showed that HcQ and Hc could bind with ganglioside. Based on challenge experiments it was revealed that HcQ, Hc and BoNT/E toxoid could give protections in mice challenged with 10<sup>2</sup>, 10<sup>4</sup> and 10<sup>5</sup> minimum lethal dose (MLD) dose of BoNT/E.

**Keywords:** Botulinum Neurotoxin; Recombinant Vaccine; Binding Domain; Synthetic Gene

## INTRODUCTION

Botulinum neurotoxins (BoNTs) produced by

*Clostridium botulinum* are the most toxic known bacterial proteins, which can be divided into seven serotypes (A to G) with similar structure, but distinct serologic properties. *C. butyricum* and *C. baratii* also produce E and F, and *C. argentinense* exclusively produces serotype G. Human botulism is commonly associated with toxin serotypes A, B, E, and F (Binz and Rummel, 2009). The BoNTs exert their action by inhibiting the release of the neurotransmitter acetylcholine at the neuromuscular junctions and synapses potentially causing death by flaccid paralysis (Yu *et al.*, 2007; Boles *et al.*, 2006; Montecucco and Schiavo, 1994). However, the role of BoNT has undergone one of the most remarkable transformations in the history of medicine due to its increasing clinical applications, which include the treatment of overactive skeletal and smooth muscles, hypersecretory and painful disorders (Adelson, 2007). BoNT is expressed as a single, 150 kD polypeptide chain that is posttranslationally nicked, forming a two-chain protein that consists of a C-terminal 100 kD heavy chain (HC) joined by a single disulfide bond to the 50 kD light chain (LC) (Dux *et al.*, 2006). HC is further delineated into a N-terminal fragment, or translocation domain (Hn), and a C-terminal fragment, or receptor-binding domain (Hc), together with single catalical domain of LC, forming three functional domains that mediate intoxication of the neuron in a defined pathway (Binz and Rummel, 2009). The Hc is responsible for binding of toxin to presynaptic membrane through multiple interactions with toxin receptors, including synaptotagmin II and synaptic vesicle protein SV2 (Dong *et al.*, 2008; Li and Singh, 1998; Nishiki *et al.*, 1996). Ganglioside binding also could facilitate interaction between toxin and its protein receptors by bringing them in close proximity, or cause a conformational change at the second receptor binding site, and is known to be mediated through the C-terminal half of Hc domain (Rummel *et al.*, 2004,

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Sharma *et al.*, 2006). Type E of BoNT (BoNT/E) displays a conserved ganglioside binding site including the key residue W1224 with the exception of the opposite lysine 1215 (Binz and Rummel, 2009). Prevention of botulism can be efficiently achieved by vaccination, which generates neutralizing antibodies against botulinum neurotoxin. Currently, the most widely available vaccines for humans are formalin-inactivated toxoid such as pentavalent toxoid (PBT). However, it is very expensive and time-consuming to produce and is hazardous during detoxification. To overcome some of the drawbacks of the toxoid vaccines, a recombinant subunit vaccine was recently investigated (Byrne *et al.*, 2000). Although complete holotoxin is required for biological toxicity, the Hc domain of several BoNT serotypes were shown to be non-toxic and capable of eliciting a protective immunity in animals challenged with homologous BoNT (Yu *et al.*, 2007; Dux *et al.*, 2006; Byrne and Smith, 2000; Clayton *et al.*, 1995). These results prompted an effort to develop a recombinant botulinum vaccine against all seven serotypes using Hc fragment as a recombinant subunit vaccine (Webb *et al.*, 2007; Boles *et al.*, 2006; Dux *et al.*, 2006; Arimitsu *et al.*, 2004; Byrne and Smith, 2000; Smith, 1998). Furthermore, prevalence of epitopic regions in extreme C terminal amino acid residues of Hc have been shown in epitope mapping studies of BoNT/A, B, and E separately (Dolimbek *et al.*, 2008; Dolimbek *et al.*, 2007; Kubota *et al.*, 1997; Atassi *et al.*, 1996). In this study, synthetic genes encoding whole binding domain (Hc) of botulinum neurotoxin type E were constructed and expressed in *E. coli*, also HcQ expression vector (Mansour *et al.*, 2010) was changed to modify its fusion protein. The abilities of the Hc and HcQ recombinant proteins and BoNT/E toxoid to elicit a protective immune response are reported herein.

## MATERIALS AND METHODS

**Chemicals and media:** Molecular biology grade chemicals and reagents and specific antibody against *Clostridium botulinum* type E neurotoxin were obtained from Sigma (St. Louis, MO, USA). pET-28a was purchased from Novagen (Madison, WI, USA). Agarose gel DNA extraction kit, chemical agents for western blotting and Ni-NTA agarose resin were obtained from Qiagen (Valencia, CA, USA).

**Construction of synthetic gene:** To improve expression levels, we designed Hc (1302 bp), encoding 433 amino acids (ca. 47 kD) of the C-terminus of the heavy chain of botulinum toxin E. Amino acid sequence of the Hc of BoNT/E (GeneBank accession number X62683) was back-translated to nucleotide sequence,

using the single most frequently used codon of *E. coli* for each amino acid. This gene were synthesized by Bio S and T (Montreal, Canada) and subsequently cloned into pET-28a to construct pET-Hc plasmids. The HcQ (282 bp-Residues 1163-1256) was subcloned into pET28a for removing the large fusion protein. The pET-HcQ was used in this study.

**Gene expression and purification of recombinant protein:** The pET-28a-Hc and pET-28a -HcQ were transformed into *E. coli* BL21 (DE3) strain (Novagen, Madison, WI, USA). The transformed colons were inoculated into 5 ml of LB medium and the culture was grown under agitation overnight at 37°C. This culture was used to inoculate 300 ml of LB medium containing 40 µg/ml kanamycin and the culture was grown at 37°C to an optical density at 600 nm (OD<sub>600</sub>) of 0.6, induced by the addition of IPTG (Isopropyl-β-D-1-Thiogalactopyranoside) to a final concentration of 1.0 mM at 30°C. The Hc and HcQ cultures were harvested by centrifugation at 5000 rpm for 10 min. The recombinant proteins were purified using Nickel-nitrilotriacetic acid (Ni-NTA) resin under native condition. Bound proteins were eluted with 1ml buffer containing 250 mM imidazole and then 500 µl of 20 mM MES buffer. The purified proteins were monitored by SDS-PAGE and concentrations of pure proteins were estimated by the Bradford protein assay method (Bradford, 1976).

**Western blot analysis:** Protein samples that were separated by SDS-PAGE were transferred to a nitrocellulose (NC) membrane in a semidry trans-blot cell. The membrane was blocked with 3% gelatin/phosphate-buffered saline for overnight at 4°C. The membrane was washed three times with Phosphate buffer saline tween-20 (PBST) and incubated with horse anti-BoNT/E IgG for 1 h at 37°C. It was then washed three times with PBST and incubated with goat anti-horse IgG conjugated with HRP for 1 h at 37°C, and was again washed three times with PBST. Finally, the membrane was developed with diaminobenzidine solution. Once the protein was visualized, the reaction was stopped with distilled water.

**Purification of BoNT/E and toxoid preparation:** *Clostridium botulinum* type E culture was added to a 10 ml cooked meat medium (Difco, USA) and cultured at 30°C for 24 h. This was carried out under biosafety precautions recommended by the national health authorities for cultivation of *C. botulinum*. 1000

ml of toxin production medium (2% proteose peptone, 1% yeast extract, 1% glucose and 0.025% sodium thioglycolate) was inoculated with the cooked meat culture of *C. botulinum* type E and incubated at 30°C for 72 h. Cells were collected and washed with 0.05 M sodium acetate buffer (pH 5.0) by centrifugation. The sediment cells were dispersed in 800 ml of 0.2 M phosphate buffer (pH 6.0). The supernatant fluid was made to 60% saturation with gradual addition of  $(\text{NH}_4)_2\text{SO}_4$  (39 g/100 ml), and stored at 4°C. The precipitate of the cell extract, collected by centrifugation, was dissolved in 35 ml of 0.05 M sodium citrate buffer (pH 5.5). The resulting solution was clarified by centrifugation and chromatographed on a DEAE-Sephadex A-50 gel. The toxic pool was dialyzed against 0.01 M phosphate buffer (pH 7.4) and applied a carboxymethyl (CM)-Sephadex CL-6B. The first protein peak, coming through essentially unretarded, was collected. L-lysine hydrochloride was added to a final concentration of 0.05 M and formalin in 0.2% every 4 days increased to a final concentration of 0.6% to purified toxin, detoxification was proceeded at 30°C. Formaldehyde was removed by dialysis against 0.01 M phosphate buffer (pH 7.4). The formalized toxin was sampled at intervals for mouse inoculation until it became completely nontoxic.

**Immunization of mice:** 2 µg from each of the recombinant purified proteins or 1 µg of formalized BoNT/E toxoid was intraperitoneally injected on days 0, 20, 34 and 48 to male Balb/C mice (20-25 g) groups of 6 animals via subcutaneous/intraperitoneal routes. Each protein was mixed with equal amount of Freund's adjuvants to a final volume of 0.4 ml. The complete adjuvant was used at the first injection and the incomplete one for the second and third injections. PBS was used for the final injection was mixed with the adjuvant served as negative control. Blood samples were collected from infraorbital plexus 14 days after the third and last injections.

**ELISA titration of sera:** ELISA was employed to titrate anti-Hc and anti-HcQ antibodies in sera of vaccinated mice. Polystyrene 96-well plates were coated with 5 µg purified Hc, HcQ or BoNT/E toxoid in 100 µl coating buffer (64 mM  $\text{Na}_2\text{CO}_3$ , 136 mM  $\text{NaHCO}_3$ , pH 9.8), and was incubated overnight at 4°C. The plates were washed with PBS/T and blocked for 45 min at 37°C with 3% gelatin in PBS/T. The plates were incubated for 30 min at 37°C with serial dilutions of sera. Following a washing step, the plates were incu-

bated for 30 min at 37°C with goat anti-mouse immunoglobulin G horseradish peroxidase (IgG-HRP, 1:12,000). The wells were then reacted with 100 µl of citrate buffer containing 0.06% (w/v) of o-phenylenediaminedihydrochloride (OPD) and 0.06% (v/v) hydrogen peroxide for 15 min at room temperature. The reaction was stopped with 100 µl of 2M  $\text{H}_2\text{SO}_4$  and the absorbance was read at 490 nm. In the same ELISA, horse anti-BoNT/E antiserum was used instead of mouse serum.

**Binding activity assay:** Binding activity of the purified Hc and HcQ to ganglioside was analyzed by ELISA. Each well of ELISA plate were coated with 1, 0.5, 0.25 or 0.125 µg of  $\text{GT}_{1b}$  (Sigma, St. Louis, MO, USA) in 100 µl methanol or 100 µl coating buffer, and incubated at 4°C overnight. After three washes in PBS/T, the plate was blocked by 3% gelatin in PBST for 1h at 37°C. All washes were for three times in PBST. After blocking and washing, 3 µg of purified Hc or HcQ in PBST were added to different wells and the plate was incubated for 2 h at 37°C on a rocker shaker. The remaining procedure was followed exactly as described above. BoNT/E was used as a positive control.

**Challenge study:** Vaccinated mice were injected intraperitoneally with  $10^2$ ,  $10^3$ ,  $10^4$  and  $10^5$  MLD (Minimum Lethal Dose) of BoNT/E, two weeks after the last booster. The challenged animals were monitored for 7 days. They were observed every 6 h for the first 2 days and twice a day thereafter. The number of deaths for each group was recorded as the endpoint. All procedures were performed in compliance with relevant laws and institutional guidelines of Shahed University.

**Statistical analyses:** ELISA results were expressed as Mean±SD (standard deviation). Significant differences between ELISA absorptions, defined as  $P < 0.05$ , were analyzed by one-way ANOVA (analysis of variance) using SPSS 16.0 software (SPSS, Chicago, IL, USA), while dilution of antisera or coated molecule (in the case of ganglioside binding assay) was considered as covariate.

## RESULTS

**Expression and purification of recombinant proteins:** Using pET-28a as expression vector, Hc and HcQ genes were expressed in *E. coli* BL21(DE3) with

a His6-tag at the N terminal. Recombinant Hc and HcQ, purified by Ni-NTA affinity chromatography, were analyzed by SDS-PAGE (Fig. 1).

The average yields of one-step purified Hc and HcQ were 3.7 and 5 mg per liter of bacterial culture, respectively. These proteins were also identified by reaction with the anti-BoNT/E in Western blots and ELISA (Figs. 2 and 3). The result as shown clearly indicates binding of Hc and HcQ to these antibodies.

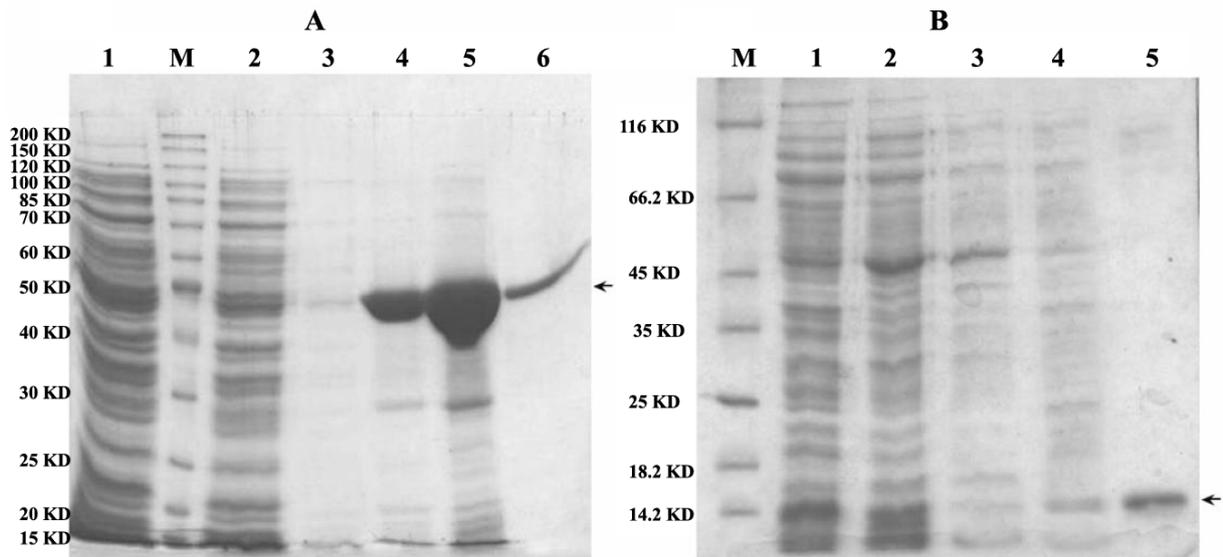
**Serum antibody titers:** Anti-Hc and HcQ antibody titers in the sera of mice bled at 2 weeks after the third (results not shown) and final vaccination were measured by ELISA using purified Hc and HcQ proteins. In both the cases, the antibody levels were significantly ( $P<0.01$ ) increased after immunization with these recombinant proteins compared to the control adjuvant-injected group (Fig.4 A). Interactions of these antisera with BoNT/E were assessed similarly. Results (Fig.4 B) showed significant binding of both antisera to the BoNT/E toxoid, compared with the control sera ( $P<0.01$ ), while there was no significant difference between anti-Hc and anti-HcQ sera ( $P>0.05$ ). Using equal amounts (5  $\mu$ g) of coated Hc, HcQ and BoNT/E toxoid, the binding of antisera (anti-Hc and anti-HcQ) to their specific recombinant antigens (Hc and HcQ respectively) did not differ significantly from their binding to the BoNT/E toxoid ( $P>0.05$ ).

**Binding activity:** In an effort to evaluate the functional state of the purified Hc and HcQ, binding of these proteins with ganglioside, as a co-receptor of BoNTs, was determined by ELISA. The results (Fig. 5) indicated significant ( $P<0.01$ ) binding of HcQ and Hc to ganglioside, compared with control. Comparing ganglioside-binding activity of two recombinant proteins in equal amounts (3  $\mu$ g protein per well), Hc showed significantly ( $P<0.05$ ) higher level of binding to GT<sub>1b</sub> than HcQ.

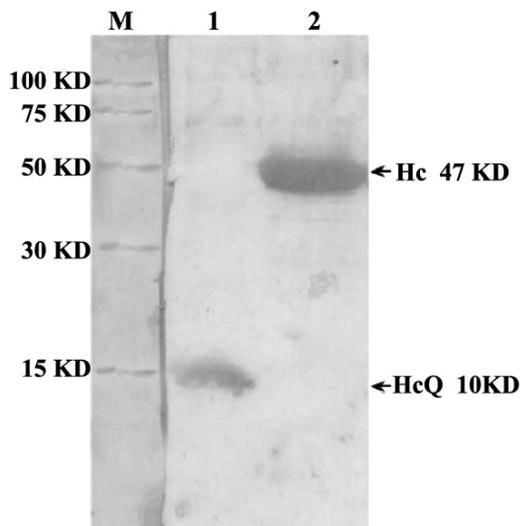
**Challenge study:** The results of challenge study showed that mice injected by HcQ, Hc and BoNT/E toxoid were fully protected from challenge with  $10^2$ ,  $10^4$  and  $10^5$  MLD doses of botulinum neurotoxin serotype E, respectively (Table 1).

## DISCUSSION

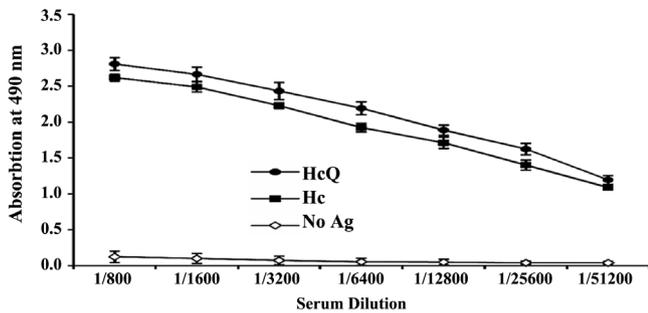
There is currently no licensed recombinant vaccine for the prevention of botulism. Limited quantities of a pentavalent toxoid vaccine granted to Investigational New Drug status in 1979 are available for individuals at risk of exposure. However, due to the difficulties and risks associated with producing toxoid vaccines (Smith *et al.*, 2004) subsequent efforts have largely



**Figure 1.** SDS-PAGE analysis of Hc A: and HcQ B: purification. Clear extract of induced cells were subjected to Ni-NTA column, followed by washing and several elutions. Proteins were visualized by Coomassie brilliant blue staining. In both (A) and (B) M indicates molecular weight marker (molecular weights are shown on the left); lane 1 is the follow-through; 2 is washing output; 3, 4, 5 and 6 indicate consecutive elution steps. Bands pertaining to Hc and HcQ are pointed by the arrows on the right of panels (A) and (B), respectively. Purified Hc were observed at fractions 6 (A) whereas and pure HcQ were obtained in fraction 5 (B).

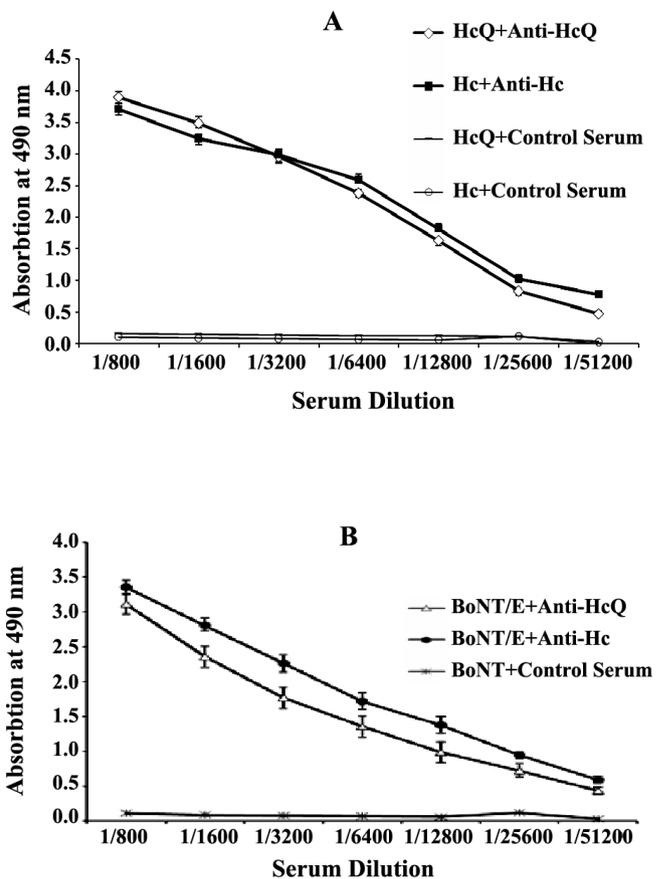


**Figure 2.** Western blot analysis of purified Hc and HcQ. Clear lysate of induced cells analysed to identify Hc and HcQ proteins, on the basis of their reactivity with anti BoNT/E antibodies. Lanes 1 and 2 are the reaction of respectively Hc and HcQ with the anti-BoNT/E antiserum. Lane M is molecular weight marker. The numbers on the left indicate the molecular mass of markers and arrows on the right indicate Hc and HcQ bands.

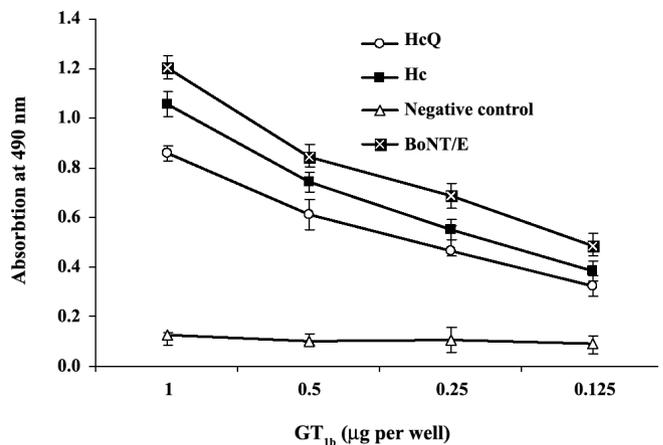


**Figure 3.** ELISA analysis of purified Hc and HcQ reaction with anti-BoNT/E. wells were coated by Hc, HcQ or no protein (as the negative control) and serial twofold dilutions of anti-BoNT/E antiserum starting at 1:800 were used. Data series are indicated by coated protein at top right. The results are expressed as absorbance at 490 nm±SD.

focused on recombinant vaccines for prophylaxis (Webb *et al.*, 2007; Dux *et al.*, 2006; Byrne *et al.*, 2000). Previous studies have shown that Hc fragment derived from BoNT/A, B, C and D can be exploited as vaccines against their respective toxin subtypes (Yu *et al.*, 2007; Boles *et al.*, 2006; Arimitsu *et al.*, 2004; LaPenotiere *et al.*, 1995). More recently Hc fragment of BoNT/E also have been used for vaccination in combination with other serotypes (Baldwin *et al.*, 2008; Ravichandran *et al.*, 2007). Although these studies clearly showed the immunogenic efficiency of Hc domains, there are some evidences suggesting that this



**Figure 4.** ELISA titration of sera from mice immunized with Hc and HcQ. A: In a single ELISA titers of anti-Hc and anti-HcQ antibodies in serum samples and B: rate of their interaction with BoNT/E toxoid were analyzed. Serial twofold dilutions of sera starting at 1:800 were used. Series are indicated by coated molecule + binding anti-sera on top-right of each panel. The results are expressed as absorbance at 490 nm±SD.



**Figure 5.** ELISA binding assay of recombinant proteins and BoNT/E to ganglioside GT<sub>1b</sub>. Wells were coated by serial twofold dilutions of GT<sub>1b</sub> and after incubation with Hc or HcQ and BoNT/E, binding were detected by anti-Hc or anti-HcQ sera or Anti BoNT/E. The results are expressed as absorbance at 490 nm±SD and series indicated by binding molecule on top-right. The results are expressed as absorbance at 490 nm±SD.

immunogenic potential may largely lean on C-terminal portion of the domain. For instance, this portion showed to contain epitopic regions at least in BoNT/A, B and E (Dolimbek *et al.*, 2008; Dolimbek *et al.*, 2007; Kubota *et al.*, 1997; Atassi *et al.*, 1996). C-terminal quarter of BoNT/A heavy chain also showed some functional properties such as binding to ganglioside, synaptotagmin and interaction with anti-BoNT/A antibodies (Sharma *et al.*, 2006). Furthermore, we previously reported that the C-terminal 93 amino acid residues of Hc BoNT/E are capable of eliciting protective immune response (Mansour *et al.*, 2010). Small molecular size of this 93aa fragment may cause some advantage including ease of production in large scales and suitability to be used in designing chimeric multivalent vaccines. In this study we compared immunogenic properties of the full and the C-terminal quarter fragment of the Hc domain of the BoNT/E. Using synthetic gene with optimized codon usage, recombinant proteins were expressed in *E. coli* at a high-level of 5 mg purified protein per liter of bacterial culture. Compared to other studies on the Hc fragments of serotypes A, C, D, E, and F produced in *E. coli* (Tavallaie *et al.*, 2004; Woodward *et al.*, 2003; Holley *et al.*, 2000; Clayton *et al.*, 1995, LaPenotiere *et al.*, 1995), the yield of recombinant protein in the current study is almost equal or in some cases higher. In addition, expression of HcQ or Hc in *E. coli* is cultured for

only 6 h at 37°C. Therefore, it is more advantageous and practical to produce and purify the recombinant protein Hc as a subunit vaccine on a large scale, using the prokaryotic expression system. As suggested by this study, the high level expression of these proteins is largely because of the designed synthetic genes and the pET expression system used. Interestingly, equal amounts of Hc and HcQ showed insignificant difference in interaction with anti-BoNT/E polyclonal antibodies (Fig. 3). Interaction of BoNT/E with anti-Hc and anti-HcQ sera also differ insignificantly (Fig. 4, B). These results indicate the comparable ability of Hc and HcQ to elicit antibodies capable of binding to BoNT/E. However, our results also showed that in terms of protection against the toxin, there is a clear difference between the two proteins. Hc vaccinated mice were protected from higher ( $10^4$  MLD) doses of the toxin (Table 1) compared with HcQ vaccinated mice ( $10^2$  MLD). Hence, because the binding activity of Hc is not completely restricted to its C-terminal quarter portion (HcQ), blocking of this region is not as protective as a complete blockage of the Hc. Stronger gangliosidebinding activity of Hc compared to HcQ (Fig. 5) further supports this concept. Center for Biologics Evaluation and Review (CBER) notifies binding of BoNTs subunit vaccines to specific receptors on cholinergic nerve cells to cause adverse neurological reactions or disorders (Byrne *et al.*, 2000). Therefore, reduced binding ability is as an advantage for HcQ. In the present work slightly stronger protection ( $10^4$  MLD) was obtained compared to the recent experiments using BoNT/E recombinant Hc domain (Baldwin *et al.*, 2008; Ravichandran *et al.*, 2007). Although 1 µg/dose of toxoid produced greater protection than 2 µg/dose of the Hc fragment (Rusnak and Smith, 2009), difficulties in the production and declining immunogenicity of the current toxoid vaccine, encouraged the idea of developing recombinant subunit vaccine against BoNTs.

**Table 1.** The protection levels with various amounts of the recombinant proteins and toxoid in the challenged mice.

Vaccination Group	Challenge dose (×MLD)	No. of animals challenged	No. of animals survived (%)
Recombinant HcQ	$10^2$	5	5 (100)
	$10^3$	5	3 (60)
	$10^4$	5	1 (20)
	$10^5$	5	0 (0)
Recombinant Hc	$10^2$	5	5 (100)
	$10^3$	5	5 (100)
	$10^4$	5	5(100)
	$10^5$	5	3 (60)
BoNT/E toxoid	$10^2$	5	5 (100)
	$10^3$	5	5 (100)
	$10^4$	5	5 (100)
	$10^5$	5	5 (100)
Adjuvant control	$10^2$	3	0 (0)
	$10^3$	3	0 (0)

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