World J Microbiol Biotechnol (2014) 30:1861–1867
DOI 10.1007/s11274-014-1609-0

ORIGINAL PAPER

High level expression, purification and immunogenicity analysis of a protective recombinant protein against botulinum neurotoxin type E

Ebrahim Valipour · Mir-Latif Moosavi · Jafar Amani · Shahram Nazarian

Received: 22 August 2013 / Accepted: 18 January 2014 / Published online: 29 January 2014

Abstract Botulinum neurotoxin type E heavy chain consists of two domains: N-terminal half as a translocation domain and C-terminal half (Hcc) as a binding domain. In this research a synthetic gene fragment encoding the binding domain of botulinum neurotoxin type E (BoNT/E-Hcc) was highly expressed in *Escherichia coli* by pGEX4T-1 vector. After purification, the recombinant BoNT/E-Hcc was evaluated by SDS-PAGE and western blot (immunoblot) analysis. Average yields obtained in this research were 3.7 mg recombinant BoNT/E-Hcc per liter of bacterial culture. The recombinant protein was injected in mice for study of its protection ability against botulinum neurotoxin type E challenges. The challenge studies showed that, vaccinated mice were fully protected against 10^4 × minimum lethal dose of botulinum neurotoxin type E.

Keywords Synthetic gene · BoNT/E · Binding domain · Western blot

Introduction

Botulinum neurotoxins (BoNTs) produced by *Clostridium botulinum* serotypes (A–G) are classified by Centers for Disease Control and Prevention (CDC) as one of the six highest-risk threat agents for bioterrorism (the "category A agents") due to their extreme potency and lethality (Hill et al. 2007). In their active form they are composed of two polypeptide chains, a heavy chain (HC, 100 kDa) and a light chain (LC, 50 kDa), held together by a disulfide bond. The C-terminal half (Hcc) (50 kDa) of the heavy chain mediates binding of the neurotoxin to specific neuronal receptors including synaptotagmin II and synaptic vesicle protein SV2, while the N-terminal half (50 kDa) enables the catalytically active light chain (LC) to translocate into the cytosol (Li and Singh 1999; Agarwal et al. 2004; Thanongsaksrikul and Chaicumpa 2011). In cytosol, the LC can cut one or more of SNARE proteins such as syntaxin, VAMP and SNAP-25 which are involved in exocytosis of neurotransmitter from pre-synaptic membrane (Basiri et al. 2010; Dressler and Adib 2005; Chen et al. 2007). The cleavage of any of the SNARE proteins results in blockage of acetylcholine release at the neuromuscular junctions, resulting in flaccid muscle paralysis.

Human botulism is commonly caused by toxin serotypes A, B, E, and F (Binz and Rummel 2009). From these strains, *C. botulinum* type E predominates in aquatic environments. Therefore, BONT/E is one of the major causative for botulism in many countries such as USA, Turkey and Iran. For example, from botulism cases reported to CDC in 2011, toxin type E accounted for 25 % and it is in second in point of causing botulism in USA. Due to a comprehensive research and study about distribution and abundance of various *C. botulinum* serotypes in Iran by Pasteur Institute of Iran, type E accounted for 72.5 %. Also, an investigation revealed that 35 % of the collected fishes from Caspian Sea were contaminated with *C. botulinum* type E (Minaei 2002; Modarres and Vahdani 2001). Protection against these hazardous toxins can be

E. Valipour (✉)
Biotechnology Department, Institute of Basic and Applied Sciences, Cukuruva University, Adana, Turkey
e-mail: va.cell@yahoo.com

M.-L. Moosavi · J. Amani · S. Nazarian
Biology Department, Faculty of Science, Shahed University, Tehran, Iran

efficiently achieved by vaccination, which generates neutralizing antibodies against botulinum neurotoxin. The most widely available botulinum vaccine is composed of formalin-inactivated crude isolates of BoNTs absorbed to aluminum phosphate and containing thimerosal as a preservative. Disadvantages of the method highlight the needs to develop more efficient approaches for vaccine development against botulism (Byrne and Smith 2000; Christina et al. 2008). So the use of binding domain of BoNTs as immunogens and vaccines against BoNTs was reported by many researches (LaPenotiere et al. 1995; Smith 1998; Lalli et al. 1999; Zhao et al. 2012). In addition to immunogenic utility, the binding domain of BoNTs has been one of the most successful and frequently used tools in neurobiology and cell biology.

Many attempts to express fragments of clostridial proteins in *Escherichia coli* have failed because of the unusually high AT content of clostridial DNA. It is possible to upgrade gene expression level of BONTs fragments in *E. coli* by optimizing the sequences according to the codon usage in *E. coli* (Karlin et al. 1998; Zdanovsky and Zdanovskaia 2000). Consequently, this research aimed to design and construction of synthetic DNA fragment that optimally encoded binding domain of neurotoxin type E, high level expression of the fragment in *E. coli* BL21DE3 and evaluation of its immunogenicity after purification.

Materials and methods

Chemicals and media

Molecular biology grade chemicals and reagents and specific antibody against *C. botulinum* type E neurotoxin were obtained from Sigma. pGEX4T-1 was purchased from Amersham Bioscience. Agarose gel DNA extraction kit, chemical agents for western blotting were obtained from Qiagen (Valencia, CA, USA).

Construction of synthetic gene

Foremost the sequence related to BoNT/E binding domain (1,302 bp) was extracted from NCBI website (Gene Bank = DQ512735.1), then C + G contents and codon usage of the wild-type BoNT/E-Hcc sequence was optimized according to the *E. coli* expression system by using websites http://eu.idtdna.com, http://www.genscript.com and http://www.entelechon.com. This optimization was carried out such that the related amino acid sequence did not undergo any change. The optimized gene fragment was synthesized by Entelechon (German). After receiving from entelechon, sequence of the synthetic gene fragment analyzed and became ensure the accuracy of sequence. Subsequently the synthetic gene fragment cloned into pGEX4T-1 to construct pGEX-HccE vector. The restriction enzyme of *Eco*RI and *XhoI* were used to preparation of the gene fragment and pGEX4T-1 vector, for ligation and cloning (Hamidi et al. 2012).

Expression and purification of the recombinant protein

The pGEX-HccE recombinant vector was transformed into *E. coli* BL21 (DE3) strain (Novagen, Madison, WI, USA) by heat shock method. The transformed host cells (colonies) were selected on LB agar plates containing 40 μg ml^{-1} ampicillin. Several of the selected colonies were cultured in LB medium and their rBoNT/E-Hcc expression was induced by IPTG (isopropyl-b-D-thiogalactopyranoside, 1 mM) and analyzed by SDS-PAGE (12 %). The colony with the most highly expression of rBoNT/E-Hcc was used to inoculate 300 ml of LB medium (pH 7.0) containing 40 μg ml^{-1} ampicillin for protein production. Then the inoculated culture was grown at 200 rpm and 37 °C until OD600 = 0.6–0.8. Subsequently the protein expression was induced by adding IPTG (1 mM) and incubation of the culture continued overnight. Then the cultures were harvested by centrifugation at 5,000 rpm for 10 min and pellet of the cells was re-suspended in 6 ml lysis buffer (100 mM NaH_2PO_4, 10 mM Tris–HCL, 8 M urea, pH = 8.0) and incubated at room temperature for 30 min to lyse cells. After the cells were completely lysed, the lysate was centrifuged at 14,000 rpm for 30 min to remove the insoluble cell debris and the supernatant was subjected to protein purification by GST purification system according to the manufacture's instruction (Amersham Bioscience). The quality and quantity of purified recombinant BoNT/E-Hcc was analyzed by SDS-polyacrylamide gel electrophoresis [SDS-PAGE (13 %)] and Bradford methods, respectively (Bradford 1976; Sambrook et al. 2001).

Western blot analysis

The recombinant protein was detected by western blot analysis using horse anti-botulinum neurotoxin type E antibody. Protein samples (rBoNT/E-Hcc) that were separated by SDS–PAGE were transferred to a nitrocellulose (NC) membrane in a semidry trans-blot cell. The membrane was incubated in the blocking buffer with gentle shaking at 4 °C overnight. Blocking buffer consisted of bovine serum albumin 3 % and phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, and 4.3 mM $Na_2HPO_4 \cdot 7\ H_2O$, pH 7.3). After decanting and discarding the blocking buffer, the membrane was incubated in a 1:3,000 dilution of horse anti-*C. botulinum* toxin type E antibody in the PBST (PBS containing 0.05 % Tween),

with gentle shaking for 1 h at room temperature. After washing the membrane with PBST for three times, each time for 5 min, blots incubated with a 1:1,000 dilution of polyclonal goat anti-horse HRP conjugate. The blot was washed three times in PBST and stained with HRP staining solution (DAB). Chromogenic reaction was stopped by rinsing the membrane twice with water (Zhou and Singh 2004; Mousavi et al. 2004; Dulal et al. 2012).

Antigenicity testing

In order to assay the recombinant protein antigenicity, the purified recombinant protein (rBoNT/E-Hcc) was mixed with complete Freund's adjuvant for initial injection and incomplete Freund's adjuvant for subsequent injections into five male Balb/c mice (6 weeks old) intraperitoneally. Each mouse received 2 μg of antigen at weeks 0, 2 and 4.

Five Balb/c mice were used as controls receiving only adjuvant. Two week after second and third injections, the animals were bled and sera were restored for ELISA analysis. ELISA plates were coated with an optimal concentration of rBoNT/E-Hcc (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 plates at 4 °C for overnight. Also, one row was incubated with coating buffer alone (no-antigen) as control. The 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 twofold dilutions in PBST, starting at 1:200, of mice serum samples were added (100 μl per well) and plates were incubated at 37 °C for 30 min. Following a washing step, plates were incubated with goat anti-mouse immunoglobulin G horseradish peroxidase (1:12,000 in PBST) at 37 °C for 30 min. The wells were then reacted with 100 μl of citrate buffer containing 0.06 % (w/v) of OPD (o-phenylenediamine dihydrochloride) and 0.06 % (v/v) hydrogen peroxide for 15 min at room temperature. The reaction was stopped with 100 μl of 2 M H_2SO_4 and the absorbance was read at 490 nm. In order to comprise the binding of sera to rBoNT/E-Hcc with binding of sera to standard BoNT/E toxin, this experiment was carried out with standard BoNT/E toxin as well (Mansour et al. 2010).

Challenge study

Two weeks after the last booster, the vaccinated mice were injected intraperitoneally with 10^2 × minimum lethal dose (MLD), 10^3 × MLD, 10^4 × MLD and 10^5 × MLD of BoNT/E. 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.

Results

Gene optimization and synthesis

Codons of BoNT/E-Hcc gene fragment were optimized for *E. coli* by Optimum GeneTM algorithm. After optimization the related amino acid sequence did not undergo any change (Fig. 1). Also after optimization, GC content of the gene increased from 22.55 to 42.53 % (Fig. 2).

Expression and purification of recombinant proteins

By using pGEX4T-1 as an expression vector, Hcc synthetic gene was expressed in *E. coli* BL21 (DE3) with a GST-tag at the N terminal. Recombinant Hcc was purified by GST-Sepharose column and analyzed by SDS-PAGE. This analysis showed a 76 kDa protein band of the recombinant Hcc (Fig. 3).

The average yields of one-step purified Hcc was 3.7 mg/l of bacterial culture. This protein was also identified by reaction with the anti-BoNT/E in western blot analysis (Fig. 4). The result clearly indicates binding of this antibody to rBoNT/E-Hcc.

Serum antibody titers

Anti-Hcc antibody titers in the sera of mice bled at 2 weeks after the two (results not shown) and final vaccination were evaluated by ELISA using purified rBoNT/E-Hcc and original BoNT/E. The antibody level was significantly increased after immunization with this recombinant protein compared to the control adjuvant-injected group (Fig. 5).

Challenge study

The results of challenge study showed that mice injected by rBoNT/E-Hcc were fully protected from challenge with 10^4 × MLD of botulinum neurotoxin type E (Table 1).

Discussion

Many studies have exploited Hcc fragment of BoNTs as vaccines against their respective toxin subtypes (Yu et al. 2007; Boles et al. 2006; LaPenotiere et al. 1995). Also, Baldwin has used Hcc fragment of BoNT/E for vaccination (Baldwin et al. 2008). Similarly in this research the binding domain of BoNT/E was used as a vaccine against BoNTs type E. For high level expression of the binding domain (Hcc) in *E. coli* BL21DE3, the related sequence was optimized according to the *E. coli* codon usage. Similarly Smith and Jensen, used this method for improving expression level of clostridial gene fragment in *E. coli*

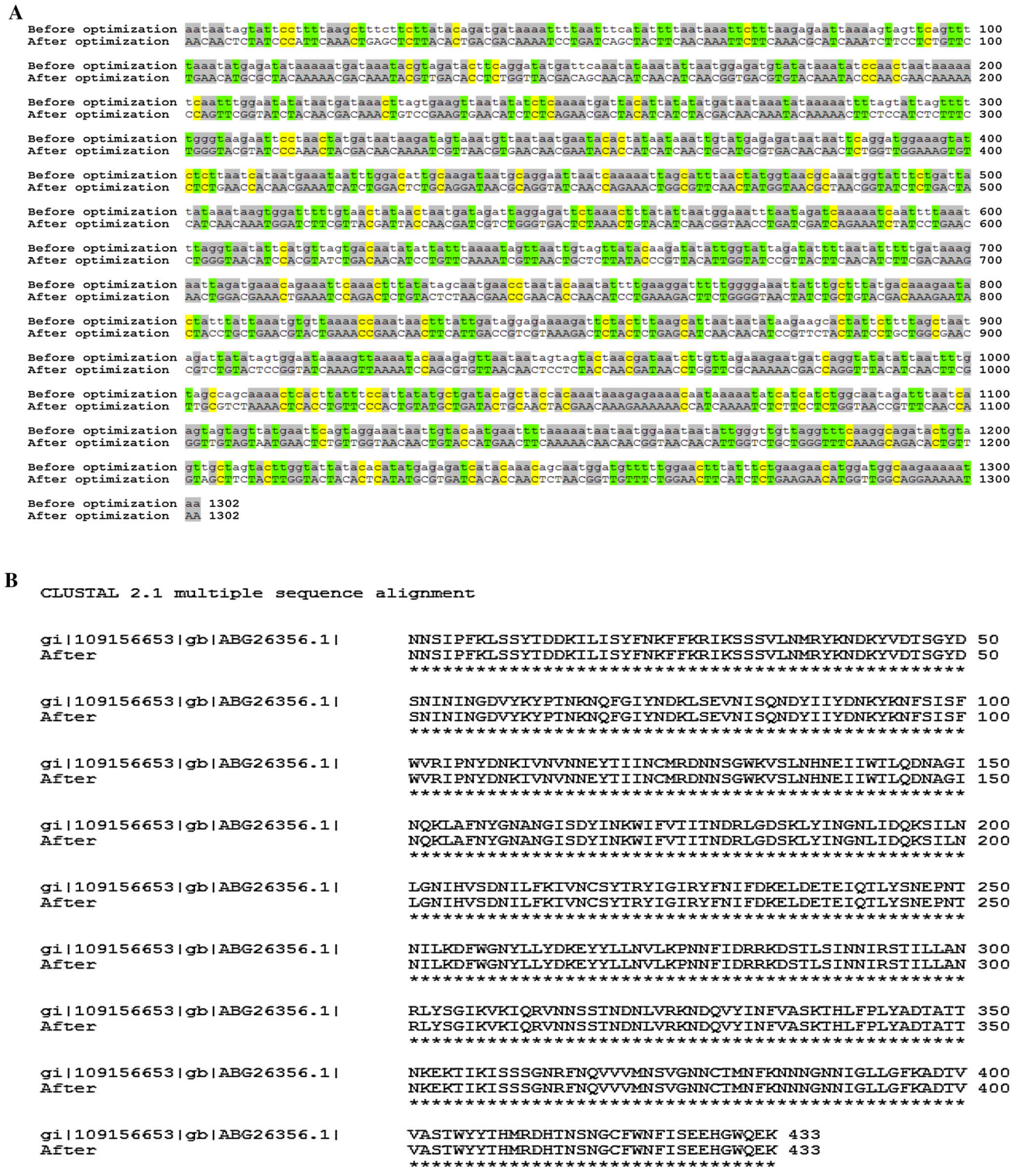

Fig. 1 Comparative alignments of original BoNT/E-Hcc (before optimization) and synthetic fragment (after optimization). **a** DNA sequence alignment, **b** amino acid sequence alignment

(Smith 1998; Jensen et al. 2003). In agreement whit this, tetanus toxin fragment C has been expressed in *E. coli* at 3–4 % cell protein while replacing of its coding sequence by synthetic sequence (which was optimized for codon usage in *E. coli*) increased the expression approximately 11–14 % (Makoff et al. 1989).

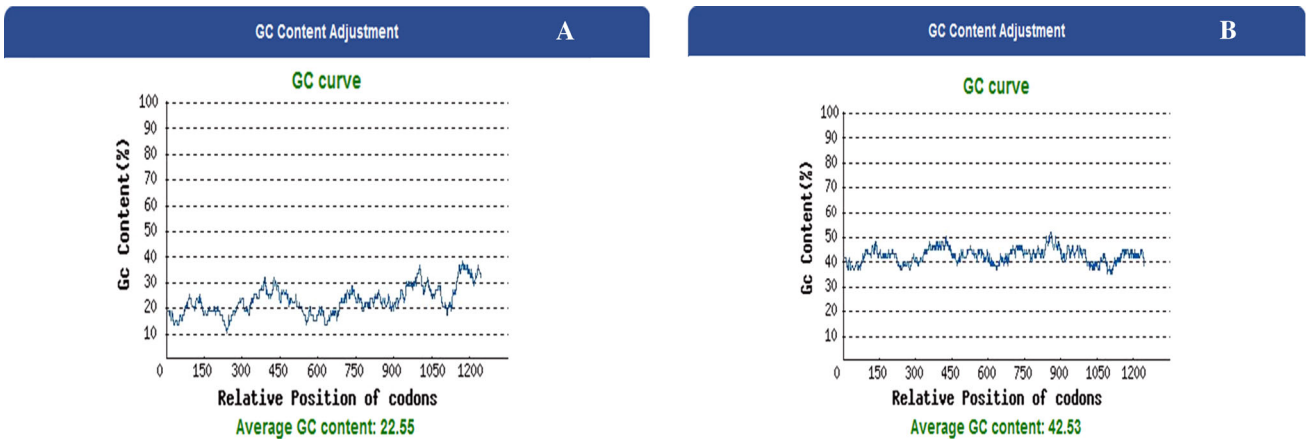

Fig. 2 GC content before **a** and after **b** gene optimization

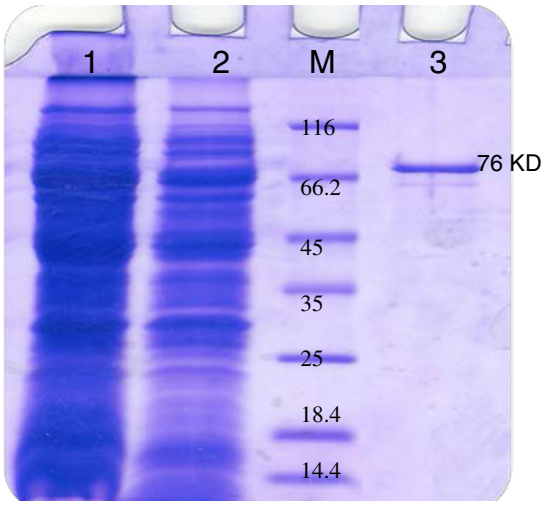

Fig. 3 SDS-PAGE analysis of BoNT/E-Hcc purification: clear extract of induced cells were subjected to GST column, followed by washing and several elutions. The proteins were visualized by Coomassie brilliant blue staining. *M* molecular weight marker, *lane 1* follow-through, *2* washing output, *3* final elution step (5 µg of purified protein was loaded in the third well)

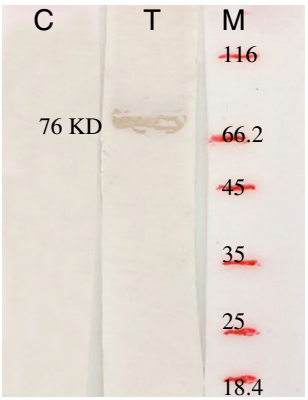

Fig. 4 Western blot analysis of rBoNT/E-Hcc (22 µg total protein from cell lysate in C and T well). The recombinant protein identified on the basis of its reactivity with anti-BoNT/E antibodies. *Lane T* total protein from cell lysate which was induced by IPTG, *lane C* total protein from cell lysate which was not induced by IPTG (Control), *lane M* molecular weight marker

In this research, the yields of production were approximately 3.7 mg of purified protein per liter of culture. This result was comparable to the results of other researches and showed the high level expression of the rBoNT/E-Hcc. For example, Woodward and her coworkers produced the BoNT/C-Hcc and BoNT/D-Hcc as vaccine candidate and obtained the yield of around 2.0 to 2.5 mg of purified proteins per liter of culture (Woodward et al. 2003). The approximate yield of 1 mg of binding domain of botulinum neurotoxin type F per one liter of culture was obtained by Holley and her/his coworkers (Holley et al. 2000). Baldwin expressed binding domain of seven serotypes (A–F) in *E. coli* and their final yield of soluble proteins (BoNT-Hcc) ranged from ~5 to 20 mg in batch culture (Baldwin et al. 2008). Also, in this research the BoNT/E-Hcc fused with GST was used as vaccine without removing GST. Similarly, Arimitsu and his coworkers, produced whole type C- and D–H-GST fusion products (HN plus HC, 100 kDa) in *E. coli*, and used these recombinant whole H products as vaccines without removing GST (Arimitsu et al. 2004).

Although toxoid vaccine produced slightly greater protection than Hcc fragment, difficulties in the production and declining immunogenicity of the toxoid vaccine encouraged the idea of developing recombinant vaccine against BoNTs (Rusnak and Smith 2009). In the present work stronger protection 10^4 × MLD was obtained compared to the former experiments using BoNT/E recombinant Hcc domain (Baldwin et al. 2008; Ravichandran et al. 2007). Interaction of original BoNT/E and rBoNT/E-Hcc

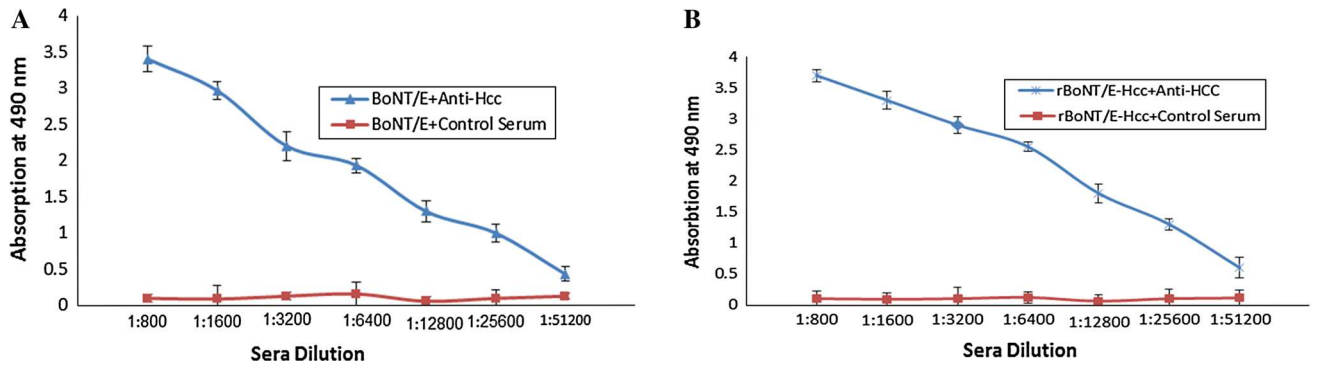

Fig. 5 ELISA analysis of purified rBoNT/E-Hcc and BoNT/E reaction with anti-Hcc (sera). **a** Rate of sera interaction with BoNT/E, **b** rate of sera interaction with purified rBoNT/E-Hcc. Unimmunized mice sera served as negative control and the results were expressed as absorbance at 490 nm ± SD

Table 1 The protection levels with the recombinant protein in the challenged mice

Vaccination group	Challenge dose (× MLD)	Number of challenged animals	Number of survived animals
Recombinant BoNT/E-Hcc	10^2	5	5 (100 %)
	10^3	5	5 (100 %)
	10^4	5	5 (100 %)
	10^5	5	3 (60 %)
Adjuvant as control	10^2	5	0 (0 %)
	10^3	5	0 (0 %)

with anti-Hcc sera showed insignificant difference in ELISA analysis and rBoNT/E-Hcc could give protections in mice challenged with 10^2, 10^3, 10^4 and 10^5 × MLD of BoNT/E.

In conclusion, rBoNT/E-Hcc can be good candidate for use as vaccine against BoNT/E and provide an effective system to study the biochemical and physical interactions involved during BoNT/E binding to nerve cells. Although the immunization with recombinant protein induced a significant protection level in mice, further studies are required to demonstrate its effectiveness in other species and possible application for use in human.

References

Agarwal R, Eswaramoorthy S, Kumaran D et al (2004) Cloning, high level expression, purification, and crystallization of the full length *Clostridium botulinum* neurotoxin type E light chain. Protein Expr Purif 34:95–102

Arimitsu H, Lee JC, Sakaguchi Y, Hayakawa Y, Hayashi M, Nakaura M, Takai H et al (2004) Vaccination with recombinant whole heavy chain fragments of *Clostridium botulinum* type C and D neurotoxins. Clin Diagn Lab Immunol 11:496–502

Baldwin MR, Tepp WH, Przedpelski A, Pier CL, Bradshaw M, Johnson EA, Barbieri JT (2008) Subunit vaccine against the seven serotypes of botulism. Infect Immun 76:1314–1318

Basiri M, Mousavi SL, Basiri H, Rasooli I (2010) An epitopic approach to designing and characterization of a multiple antigenic polypeptide against botulinum neurotoxins A and E. World J Microbiol Biotechnol 26:1659–1666

Binz T, Rummel A (2009) Cell entry strategy of clostridial neurotoxins. J Neurochem 109:1584–1595

Boles J, West M, Montgomery V, Tammariello R, Pitt ML, Gibbs P, Smith L, LeClaire RD (2006) Recombinant C fragment of botulinum neurotoxin B serotype (rBoNTB (HC)) immune response and protection in the rhesus monkey. Toxicon 47:877–884

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254

Byrne MP, Smith LA (2000) Development of vaccines for prevention of botulism. Biochimie 82:955–966

Chen S, Kim JJ, Barbieri JT (2007) Mechanism of substrate recognition by botulinum neurotoxin serotype A. J Biol Chem 282:9621–9627

Christina LP, William HT, Marite B et al (2008) Recombinant holotoxoid vaccine against botulism. Infect Immun 76(1):437–442

Dressler D, Adib SF (2005) Botulinum toxin: mechanisms of action. Eur Neurol 53:3–9

Dulal S, Ban B, Yang GH, Jung HH (2012) Cloning, expression, purification, and characterization of *Clostridium botulinum* neurotoxin serotype F domains. Nepal J Biotechnol 2(1):1–15

Hamidi B, Ebrahimi F, Abbas Hajizadeh A, Keshavarz AH (2012) Fusion and cloning of the binding domains of botulinum neurotoxin type A and B in *E. coli* DH5α. Eur J Exp Biol 2(4):1154–1160

Hill KK, Smith TJ, Helma CH, Ticknor LO (2007) Genetic diversity among botulinum neurotoxin-producing clostridial strains. J Bacteriol 189(3):818–832

Holley JL, Elmore M, Mauchline M, Minton N, Titball RW (2000) Cloning, expression and evaluation of a recombinant sub-unit vaccine against *Clostridium botulinum* type F toxin. Vaccine 19:288–297

Jensen MJ, Smith TJ, Ahmed SA, Smith LA (2003) Expression, purification, and characterization of the botulinum neurotoxin A catalytic domain and its C-terminal fusion variants. Toxicon 41:670–691

Karlin S, Mrazek J, Campbell AM (1998) Codon usages in different gene classes of the *Escherichia coli* genome. Mol Microbiol 29:1341–1355

Lalli G, Herreros J, Osborne SL, Montecucco C, Rossetto O, Schiavo G (1999) Functional characterization of tetanus and botulinum neurotoxins binding domain. J Cell Sci 112:2715–2724

LaPenotiere HF, Clayton MA, Middlebrook JL (1995) Expression of a large, nontoxic fragment of botulinum neurotoxin serotype A and its use as an immunogen. Toxicon 33:1383–1386

Li L, Singh BR (1999) Structure–function relationship of clostridial neurotoxins. J Toxicol Toxin Rev 18:95–112

Makoff AJ, Oxer MD, Romanos MA, Fairweather NF, Ballantine S (1989) Expression of tetanus toxin fragment C in *E. coli*: high level expression by removing rare codons. Nucleic Acids Res 17:10191–10202

Mansour AA, Mousavi SL, Rasooli I, Nazarian S, Amani J, Farhadi N (2010) Cloning, high level expression and immunogenicity of 1163–1256 residues of C-terminal heavy chain of *C. botulinum* neurotoxin type E. Biologicals 38:260–264

Minaei ME (2002) production and purification of botulinum neurotoxin type E and preparation of antibody against the toxin. Dissertation, University of Imam Hosein, Tehran, Iran

Modarres SH, Vahdani P (2001) Epidemiologic study and determination of botulism causative types of *Clostridium botulinum* strains in Iran. In: Fourth congress of infectious diseases in Iran, pp 411–412

Mousavi ML, Montaser Kouhsari S, Nazarian S, Rasooli I, Amani J (2004) Cloning, expression and purification of *Clostridium botulinum* neurotoxin type E binding domain. Iran J Biotechnol 2:183–188

Ravichandran E, Al-Saleem FH, Ancharski DM, Elias MD, Singh AK, Shamim M, Gong Y, Simpson LL (2007) Trivalent vaccine against botulinum toxin serotypes A, B, and E that can be administered by the mucosal route. Infect Immun 75:3043–3054

Rusnak JM, Smith LA (2009) Botulinum neurotoxin vaccines: past history and recent developments. Hum Vaccin 5:794–805

Sambrook J, Fritsch EF, Maniatis T (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Smith LA (1998) Development of recombinant vaccines for botulinum neurotoxin. Toxicon 36:1539–1548

Thanongsaksrikul J, Chaicumpa W (2011) Botulinum neurotoxins and botulism: a novel therapeutic approach. Toxins 3:469–488

Woodward LA, Arimitsu H, Hirst R, Oguma K (2003) Expression of HC subunits from *Clostridium botulinum* types C and D and their evaluation as candidate vaccine antigens in mice. Infect Immun 71:2941–2944

Yu YZ, Sun ZW, Wang S, Yu WY (2007) High-level expression of the Hc domain of *Clostridium botulinum* neurotoxin serotype A in *Escherichia coli* and its immunogenicity as an antigen. Sheng Wu Gong Cheng Xue Bao 23:812–817

Zdanovsky AG, Zdanovskaia MV (2000) Simple and efficient method for heterologous expression of clostridial proteins. Appl Environ Microbiol 66(8):3166–3173

Zhao H, Nakamura K, Kohda T, Mukamoto M, Kozaki S (2012) Characterization of the monoclonal antibody response to botulinum neurotoxin type A in the complexed and uncomplexed forms. Jpn J Infect Dis 65:138–145

Zhou Y, Singh BR (2004) Cloning, high-level expression, single-step purification, and binding activity of His6-tagged recombinant type B botulinum neurotoxin heavy chain transmembrane and binding domain. Protein Expr Purif 34:8–16