Protein Expression and Purification 146 (2018) 51–60

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

High level expression and immunochemical characterization of botulinum neurotoxin type F light chain

Ritika Chauhan[a], Vinita Chauhan[a], Mula Kameshwar Rao[b], Dilip Chaudhary[c], Sameer Bhagyawant[d], Ram Kumar Dhaked[a,*]

[a] Biotechnology Division, India
[b] Division of Pharmacology and Toxicology, Defence Research & Development Establishment, Jhansi Road, Gwalior 474002, India
[c] Defence Laboratory, Ratanada, Jodhpur 342011, India
[d] School of Studies in Biotechnology, Jiwaji University, Gwalior 474001, India

ARTICLE INFO

Keywords:
Clostridium botulinum
Botulinum neurotoxins type F (BoNT/F)
Surface plasmon resonance (SPR)
Vesicle associated membrane protein (VAMP-2)
Small molecule inhibitors (SMIs)

ABSTRACT

Botulinum neurotoxins (BoNTs) are the most toxic biological substances known. Their potential use as biological warfare agent results in their classification as category A biowarfare agent by Centers for Disease Control and Prevention (CDC), USA. Presently, there are no approved detection system and pharmacological treatments for BoNT intoxication. Although a toxoid vaccine is available for immuno-prophylaxis, vaccines cannot reverse the effect of pre-translocated toxin. Direct handling of the live BoNTs for developing detection and therapeutics may pose fatal danger.

This concern was addressed by purifying the recombinant catalytically active light chain of BoNT/F. BoNT/F-LC gene was amplified from the genomic DNA using specifically designed primers and expressed in *Escherichia coli*. Expression and purification profile were optimized under different conditions for biologically active light chain production. Specific polyclonal antibodies generated against type F illustrates *in vivo* neutralization in mice and rabbit. These antibodies play key role in conceiving the development of high throughput SPR based detection system which is a highly precise label free technique for protein interaction analysis.

The presented work is first of its kind, signifying the production of highly stable and active rBoNT/F-LC and its immunochemical characterization. The study aids in paving the path towards developing a persistent detection system as well as in presenting comprehended scheme for *in vitro* small molecule therapeutics analysis.

1. Introduction

The botulinum neurotoxins (BoNTs) are the endopeptidases produced by Gram positive, rod shaped, motile, non-encapsulated, spore forming anaerobic bacteria *Clostridium botulinum, C. butyricum* and *C. baratii* as ~150 kDa single polypeptide chain [1,2]. These neurotoxins are the most toxic substances known to humankind and have been evidently related to neuroparalytic illness 'botulism', which could prove fatal in course of time owing to respiratory failure [3]. Instead of rare natural occurrence of botulism, the botulinum neurotoxins have been classified as Category A biological threat agents by Centers for Disease Control and Prevention (CDC), USA [4], considering the ease in production and dissemination, unavailability of reliable treatment and limited supportive care facility [5]. There are seven antigenically distinct classes denoted BoNT/A-G [2] and very recently discovered yet debatable eighth serotype BoNT/H [6]. One of the discoveries recognized it as a novel serotype while further study proved it as subtype of pre-existing serotypes [7]. Even though the reported human cases are of serotypes A, B, E, and F; interestingly, all the serotypes may potentially infect human. All toxinotypes inhibit acetylcholine release from nerve terminals but vary significantly in their binding receptors and intracellular target proteins [8,9].

These toxins are proteolytic cleaved into dichain consisting of a ~100 kDa heavy chain (HC) and a ~50 kDa light chain (LC) that remain associated in native state by disulfide linkage. The ~100 kDa HC is composed of the cell receptor binding carboxy terminal (H_C) and translocation amino terminal (H_N) domains and the ~50 kDa LC act as catalytic domain. The H_C binds to the receptor present on the unmyelinated presynaptic membrane of the cholinergic cells leading to the internalization of the neurotoxin by endocytosis. The translocation of the LC takes place through a channel arbitrated by the H_N domain from the endosome to the cell cytosol [10]. The Zn endopeptidase activity of the LC leads to the proteolytic cleavage of either of the Soluble N-ethylmaleimide sensitive factor Attachment Receptor (SNARE)

* Corresponding author.
E-mail address: rkdhaked@drde.drdo.in (R.K. Dhaked).

https://doi.org/10.1016/j.pep.2018.01.014
Received 1 December 2017; Received in revised form 16 January 2018; Accepted 27 January 2018
Available online 31 January 2018
1046-5928/

proteins i.e. Synaptosome associated protein (SNAP-25), VAMP/synaptobrevin and syntaxin that prevents the exocytosis of acetylcholine by inhibiting neurotransmitter vesicle fusion with the plasma membrane [11].

Presently, mechanical ventilation is the most prominent treatment procedure in BoNTs intoxication [5]. Heptavalent botulism antitoxin (HBAT) equine is provided by the CDC for serotypes A-G and is in use in the United States since 2008 but absence of well-organized studies on its ability to neutralize various subtypes of BoNTs denigrates its efficacy [12]. Human botulism immune globulin intravenous (BIG-IV), a human antitoxin available from the California department of health services against serotypes A-E reduces recovery time when administered early in infants [13]. The antitoxin treatment has limitations like BoNTs present in blood are neutralized while intoxicated nerve cells remain unaffected and equine antitoxins pose possible side-effect of serum-sickness [12,13]. Alternatively, vaccination of mass at-risk workers using an investigational pentavalent botulinum toxoid (PBT) and new recombinant vaccine (rBV A/B-40) [14,15] could be performed. However, the implication seems farfetched considering the clinical utility of BoNTs in various medical conditions. In the recent proceedings to find an effective medication, target based drug discovery has been presented as promising approach. There are several steps in the intoxication process that could be the target of inhibitors, however, clinical presentation of botulism occurs after the LC activity in the cytosol. Therefore, it is desirable to find small molecule inhibitor that can neutralize internalized LC enzyme. There have been numerous reports on finding SMI to BoNT/A [16–23], few on inhibitors of BoNT/B [24–27,31] & BoNT/E [28–31] and one on BoNT/F [31]. Bing et al. (2010), have reported inhibitors that were effective against both BoNT/A and BoNT/B [32].

Our concern has been to deal with finding of SMIs against overlooked serotype (BoNT/F) which even though less toxic than BoNT/A is equally perilous in bio-warfare scenario. The extensive research requires direct handling of the neurotoxins which brings the researcher at high risk. We have tried to address this issue by cloning, expressing and purifying highly active recombinant light chain of type F i.e. BoNT/F-LC in native condition to carry on evaluation of SMI. There are only two reports till date dealing with expression and purification of BoNT/F-LC without mentioning their biological activity [33,34]. In present study, we have purified recombinant BoNT/F-LC and its enzymatic activity determined through highly precise endopeptidase assay using in house produced rGST-VAMP-2 [35] (substrate of BoNT/F). The antibodies raised against rBoNT/F-LC in mice and rabbit showed higher level of specificity and generated protective immune response in mice as observed in cross protection assay. The Ig isotyping displayed the high level of IgG1 and IgG2 subtypes. Affinity and specificity of these antibodies is also analysed through surface plasmon resonance (SPR). These antibodies could play role in developing future detection assay and the active rBoNT/F-LC will be used for the development of the effective medical countermeasures.

2. Material and methods

Yeast extract and tryptone for bacterial culture media were obtained Difco Laboratories, USA. HRP-labelled secondary antibodies, rabbit anti mouse immunoglobulins (P0161) & goat anti rabbit immunoglobulins (P0448) were purchased from DAKO, Denmark. Monoclonal anti-polyhistidine antibody produced in mouse (H1029), anti-glutathione-S-transferase (GST)–peroxidase conjugate antibody produced in rabbit (A7340), anti-vesicle-associated membrane protein 2 antibody produced in rabbit (V1389) were acquired from Sigma Aldrich, USA. Mouse monoclonal antibody isotyping reagents (ISO2), chicken anti-goat IgG antibody, HRP conjugate (AP163P) were also purchased form Sigma Aldrich, USA. Chemiluminescent peroxidise substrate (CPS350), 3,3′-Diaminobenzidine (DAB), copper (II) sulphate pentahydrate 4% solution, bicinchoninic acid solution, and other required chemicals were obtained from Sigma Aldrich, USA. Genomic DNA isolation QIAquick gel extraction kit (28,706), DNeasy blood & tissue kit (69,506) and plasmid DNA purification QIAprep spin miniprep kit (27,106) were purchased from Qiagen, Germany. *Escherichia coli* host, vector (pQE-30UA), glutathione HiCap matrix (30,900) and Ni-NTA Agarose (30,210) were also purchased from Qiagen, Germany. Pierce unstained protein molecular weight marker, (SM0431), DNA Ladder (SM0313) were purchased from Fermentas, USA. Novex prestained protein marker (LC5800) and polymerase chain reaction kit (10,342,020) were purchased from Invitrogen, USA. Primers were synthesized from Microsynth, Switzerland. The recombinant GST-VAMP-$2_{1–96}$ vector was kindly provided by Dr Christian Leveque, INSERM, France.

2.1. Cloning BoNT/F-LC gene and expression of recombinant protein

Trypticase-peptone-yeast extract-glucose (TPYG) media was used to grow culture of *C. botulinum* type F anaerobically at 37 °C and genomic DNA was isolated from the overnight (O/N) grown culture using DNA extraction. BoNT/F-LC forward primer 5′- ATGCCAGTTGCAATAAAT AGT -3' (Tm, 54.8 °C) and BoNT/F-LC reverse primer 5′- CTATTATTT ACTCTAATGCAT -3' (Tm, 54.6 °C) were designed and used to amplify full length BoNT/F-LC gene through PCR. The gel-extracted amplicon (~1352 bp), was cloned in pQE-30UA expression vector into electrocompetent *E. coli* SG13009. In order to check the orientation of LC gene in pQE-30UA vector, PCR was performed with pQE-30UA reverse primer (5′-GTTGTGAGGTCATTACTG-3′) and LC forward primer. One clone was selected and sequenced by automated sequencer ABI 310 sequencer (Applied Biosystem, USA). The mother culture of recombinant BoNT/F-LC was cultured and various optimization experiments were carried out with respect to different media; Luria Bertani (LB), Super broth (SB) and 2x Yeast extract tryptone (2YT)); isopropyl thio-galactoside (IPTG) concentration (0.25, 0.5, 1 and 1.25 mM); temperature (37, 25 and 18 °C) and time after induction (1, 2, 3, 4, 5 h & O/N) (data not shown). The final optimized condition was taken as induction at OD_{600nm}–0.7 by 0.5 mM IPTG and grown in LB medium O/N at 18 °C at 200 rpm. Cells were harvested and level of expression and localization of protein was examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

2.2. Purification of recombinant BoNT/F-LC

Purification of 6X His-tagged recombinant protein was carried out in native condition from the supernatant obtained after cell lysis (French press) of culture pellet grown O/N at 18 °C at 200 rpm induced using 0.5 mM IPTG at OD_{600} ~ 0.7. Ni-NTA affinity chromatography was used for the purification. Initially, we tried purification by equilibrating through lysis buffer (300 mM NaCl in 50 mM sodium phosphate buffer, pH 8.0), but nonspecific bound proteins were found. Various concentration of triton X-100, 2-Mercaptoethanol (BME) and imidazole were used to identify optimum condition for Ni-NTA binding to determine the most suitable combination for purification. Finally, Ni-NTA agarose column was pre-equilibrated with lysis buffer containing triton X-100 (0.1%), BME (7.15 mM), and imidazole (10 mM) to avoid non-specific binding. The supernatant was allowed to interact with pre-equilibrated Ni-NTA agarose for 2 h and the protein was eluted using varied concentrations of imidazole (75, 100, 150, 250 mM). To increase stability and to avoid protein denaturation the elution was performed in buffer containing 10% glycerol as reported earlier [27]. The elutes were then analysed on 10% SDS-PAGE and confirmed through Western blot. Western blot was performed by transferring the protein electrophoretically to the nitro cellulose (NC) membrane. After 2 h blocking in 3% bovine serum albumin (BSA, fraction V) in Phosphate Buffer Saline (PBS) at room temperature (RT), the NC membrane was incubated in anti-His in 1:2000 dilution for 1 h. The membrane was washed thrice