Original Article

Production and Characterization of Recombinant Light Chain and Carboxyterminal Heavy Chain Fragments of Tetanus Toxin

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Abstract

Background: Light chain (LC) and heavy chain carboxyterminal subdomain (H_{CC}) fragments are the most important parts of tetanus neurotoxin (TeNT) which play key roles in toxicity and binding of TeNT, respectively. In the present study, these two fragments were cloned and expressed in a prokaryotic system and their identity was confirmed using anti-TeNT specific polyclonal and monoclonal antibodies.

Methods: LC and H_{CC} gene segments were amplified from Clostridium tetani genomic DNA by PCR, cloned into pET28b(+) cloning vector and transformed in *Escherichia coli* (*E. coli*) BL21(DE3) expression host. Recombinant proteins were then purified through His-tag using Nickel-based chromatography and characterized by SDS-PAGE, Western blotting and ELISA techniques.

Results: Recombinant light chain and H_{CC} fragments were successfully cloned and expressed in (*E. coli*) BL21 (DE3). Optimization of the induction protocol resulted in production of high levels of H_{CC} (~35% of total bacterial protein) and to lesser extends of LC (~5%). Reactivity of the His-tag purified proteins with specific polyclonal and monoclonal antibodies confirmed their renatured structure and identity.

Conclusion: Our results indicate successful cloning and production of recombinant LC and H_{CC} fragments of TeNT. These two recombinant proteins are potentially useful tools for screening and monitoring of anti-TeNT antibody response and vaccine production.

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Keywords: Fragment C, Light chain, Monoclonal antibody, Tetanus toxin

Introduction

Tetanus is a highly fatal disease caused by a neurotoxin of a gram positive and anaerobic bacterium of the Clostridium genous, *Clostridium tetani* [1]. TeNT and seven botulinum neurotoxins (BoNT/A-G) make the family of clostridial neurotoxins (CNTs), which are exclusively responsible for neuroparalytic syndromes of tetanus and botulism [2].

TeNT is produced as a single polypeptide (approximately 150 *kDa*) and subsequently cleaved to a two-chain active holotoxin, in which a 50 *kDa* N-terminal Light Chain (LC) and a 100 *kDa* C-terminal Heavy Chain (HC) are linked by a single disulphide bond [3,4].

Tetanus toxin light chain holds the HEXXH zinc protease consensus motif and acts as a toxic part of toxin and zinc-dependent endopeptidase [5,6]. Tetanus toxin HC is composed of the aminoterminal half (HN~50 *kDa*) which is important for LC translocation and the carboxyterminal half (HC or fragment C~50 *kDa*) which holds the key amino acid residues responsible for the binding activity of the CNTs [7]. Fragment C or the carboxy-terminal half of HC is further subdivided in two subdomains: the proximal H_{CN} subdomain and the extreme carboxy subdomain, H_{CC}. H_{CC} subdomain has a key role in binding of CNTs to the neuron gangliosides [8,9].

All CNTs cleave the specific family of proteins integral to the exocytotic process [the soluble N-ethyl-maleimide-sensitive fusion (NSF) protein attachment receptor (SNARE) proteins] [10] and block neurotransmitter release and neurosecretion. Among the CNTs, TeNT inhibits the release of inhibitory neuro-transmitter glycine and γ-aminobutyric acid through proteolytic cleavage of the neuronal SNARE protein synaptobrevin/ VAMP2 [5,11,12].

The humoral immune response plays a crucial role against tetanus and antibodies directed against multiple epitopes of TeNT involved in toxin neutralization [13]. In this regard, production and characterization of different parts of tetanus toxin (especially LC and H_{CC} subdomains) are important for understanding the intoxication mechanisms and also for production of neutralizing monoclonal antibodies.

Materials and Methods

Bacterial strains

E. coli strains JM109, Top10F' and BL21 (DE3) (Novagen, Darmstadt, Germany) were cultured in LB agar containing 0.5% *w/v* yeast extract (Merck KGaA, Darmstadt, Germany), 1% *w/v* peptone (Merck KGaA, Darmstadt, Germany), 0.6% *w/v* NaCl and 1.5% *w/v* agar (Merck KGaA, Darmstadt, Germany). LB broth medium components were similar to LB agar except agar.

Construction and expression of the recombinant proteins

TeNT light chain and H_{CC} subdomain of heavy chain were amplified from *Clostridium tetani* genomic DNA for construction of the recombinant proteins. Polymerase Chain Reaction (PCR) was performed using specific primers containing BamHI and HindIII restriction sites in both ends (shown as bold sequences): 5-**GGATCC**TATGCCAATAACCAT AAATAATTTTAG-3 as sense and 5-**AAGCTT** TGCAGTTCTATTATATAAATTTTCTC-3 as antisense for LC and 5-**GGATCC**TTTATCTA TAACCTTTTTAAGAGACTTC-3 as sense and 5-**AAGCTT**ATCATT TGTCCATCCTTCATCT G-3 as anti-sense for H_{CC}.

PCR reactions were performed in 25 *μl* volumes using 1 unit/reaction pfu DNA polymerase (Fermentas, Moscow, Russia), 2.5 *μl* of 10 X PCR buffer, 1.5 *μl* of 25 *mM* MgSO4, 1.0 *μl* of dNTPs (10 *mM*) (Roche Applied Science, Indianapolis, USA), and 1 *pmol* of sense and anti-sense primers, respectively. Each amplification reaction underwent initial denaturation at 94°*C* for 5 *min* followed by 40 cycles at 94°*C* for 1 *min*, 54.7°*C* (light chain) and 57°*C* (H_{CC}) for 1 *min* and 72°*C* for 1 *min* and 10 *min* at 72°*C* for the final extension. PCR products were finally visualized by electrophoresis over 1% agarose gel containing ethidium bromide. PCR products were extracted using the GF-1 Nucleic Acid Extraction Kit (Vivantis, Selangor Darul Ehsan, Malaysia). Gel-purified PCR products were directly cloned in pGEMT-easy cloning vector (Promega, Madison, USA) and transformed into *E.coli* JM109 or TOP10F' competent cells. Sequencing of selected clones was performed using a BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA), and T7 and SP6 primers. After confirmation of the selected clones by sequencing, inserts were digested with restriction endonucleases BamHI and HindIII (Fermentas, Moscow, Russia) and ligated in pET28b(+) expression vector (Merck Millipore, Darmstadt, Germany). pET28b(+) light chain or H_{CC} constructs were transformed into (*E. coli*) BL21 (DE3) expression host. Positive clones were selected by colony-PCR. The colony-PCR was performed in 25 cycles using Taq DNA polymerase instead of pfu DNA polymerase. After confirmation by colony-PCR, transformed cells were grown in LB broth containing 50*µg/ml* kanamycin; 1-5*mM* IPTG (1, 2, 3, 4 and 5 *mM*) were used to induce protein production and finally after 2, 4 and 16 *hr* of incubation at 37°*C*, cells were harvested by centrifugation at 2000 *g* for 30 *min* at 4*°C*.

Purification of the recombinant proteins

Purification of recombinant proteins was performed using Nickel-Nitrilotriacetic Acid (Ni-NTA) chromatography column (Qiagen, Germantown, Maryland, USA) under denaturing condition. In this regard, harvested bacterial pellets containing inclusion bodies were solubilized in 20 *ml* of lysis buffer (100 *mM* NaH2PO4, 100 *mM* NaCl, 30 *mM* TrisHCL, pH=8) and incubated on ice for 1 *hr*. This solution was continuously sonicated at 70% amplitude for 15 *min* for cell destruction and then centrifuged at 12000 *g* for 10 *min* at 4*°C*.

Pellets were resuspended in buffer A (100 *mM* NaH2PO4, 50 *mM* NaCl, 10 *mM* Tris-HCL, 30 *mM* imidazole, 8 *M* urea, pH=8) and incubated at room temperature for 1 *hr*. After centrifugation at 18000 *g*, for 30 *min* at 4*°C*, supernatants were applied as starting materials on Ni-NTA agarose (Qiagen, Germantown, Maryland, USA) column equilibrated with buffer A.

Refolding process was accomplished using a continuous declining gradient of urea concentration from 8 *M* to zero for 3 *hr*. Subsequently, buffer B (100 *mM* NaH2PO4, 50 *mM* NaCl, 10 *mM* Tris-HCL, 80 *mM* imidazole, pH=8) was used to detach nonspecific proteins from the column. Elution of target proteins was performed using buffer C (100 *mM* NaH2PO4, 50 *mM* NaCl, 10 *mM* Tris-HCL, 500 *mM* imidazole, pH=8). Finally, purity of target proteins was checked using SDS-PAGE and protein concentrations were determined using BCA colorimetric assay kit (Pierce, Rockford, IL, USA).

Western blot analysis

Non-reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of recombinant LC and H_{CC} was carried out on a 12% polyacrylamide gel. Thereafter, proteins were transferred to PVDF or Nitrocellulose membranes (Merck KGaA, Darmstadt, Germany) at 100 *V* for 35 *min* using an electroblot system (BioRad, Hercules, California, USA).

After blocking the membrane with blocking buffer (PBS-T+5% non-fat skim milk) overnight at 4*°C*, and then washing four times with PBS-T, human anti tetanus toxin polyclonal antibodies (prepared in our lab) were added at 10 *µg/ml* and the membrane was incubated with gentle rocking at RT for 1.5 *hr*. The membrane was then gently washed four times with PBS-T. After washing, HRP-conjugated sheep anti-human Ig solution (prepared in our lab) was added to membranes and incubation was performed under the same conditions of the primary antibodies. Finally each blot was developed using ECL detection kit (GE Healthcare Life Sciences, Uppsala, Sweden).

Characterization of recombinant H_{CC} and LC proteins by ELISA

For final confirmation of the identity of recombinant H_{CC} and LC proteins, ELISA was carried out using human anti-TeNT polyclonal and monoclonal antibodies, as described elsewhere [14]. Briefly, ELISA plates were coated with appropriate concentration of recombinant H_{CC} and LC (10 *µg/ml*), tetanus toxin (10 *µg/ml*) and toxoid (10 *µg/ml*) (Razi Vaccine and Serum Research Institute, Karaj, Iran) in Phosphate Buffer Saline (PBS, 0.15 *M*, pH=7.2) overnight at 4*°C*. After washing, the plate was blocked using blocking buffer (PBS-Tween 20 containing 3% non-fat skim milk) at 37*°C* for 1.5 *hr*. After blocking and washing, 100 *µl* of 1 *µg/ml* purified human polyclonal and mouse monoclonal antibodies were added separately and incubated for 1.5 *hr* at 37*°C*. Appropriate dilution of HRP-conjugated rabbit anti-human and rabbit anti-mouse (prepared in our lab) was subsequently added and the reaction was revealed with 3, 3´,5,5´-Tetramethylbenzidine (TMB) substrate. Finally, the reaction was stopped with 20% H_2SO_4 and the optical density (OD) was measured by a multiscan ELISA reader (Organon Teknika, Boxtel, Belgium) at 450 *nm*.

Results

Construction and expression of recombinant light chain and H_{CC} proteins

LC and H_{CC} were amplified from *Clostridium tetani* genomic DNA by PCR. The amplified LC and H_{CC} PCR product sizes, 1371 and 621 *bp* respectively, were confirmed using agarose gel electrophoresis (Figure 1A). Sequencing of both gene segments showed complete homology with the reference genome sequence of *Clostridium tetani* Harvard strain (NCBI Gene Bank accession number: M12739), (data not presented). Both genes were then cloned into pET28b(+) expression vector and the constructs were verified by sequencing and digestion using BamHI and HindIII restriction endonucleases (Figure 1B) before transformation into (*E. coli*) BL21(DE3) expression host. To optimize the induction protocol of the two recombinant proteins, different concentrations of IPTG (1, 2, 3, 4 and 5 *mM*), incubation times (from 1-16 *hr*) and incubation temperatures (25°*C* and 37°*C*) were applied. High levels of expression were obtained for H_{CC} using 1 *mM* IPTG at 25°*C* and 8 *hr* of induction time in (*E. coli*) BL21 (DE3), (Figure 2A). Lower levels of expression were achieved for LC (Figure 2B) with no significant improvement despite changing all parameters of the induction protocol and application of different *E. coli* hosts including

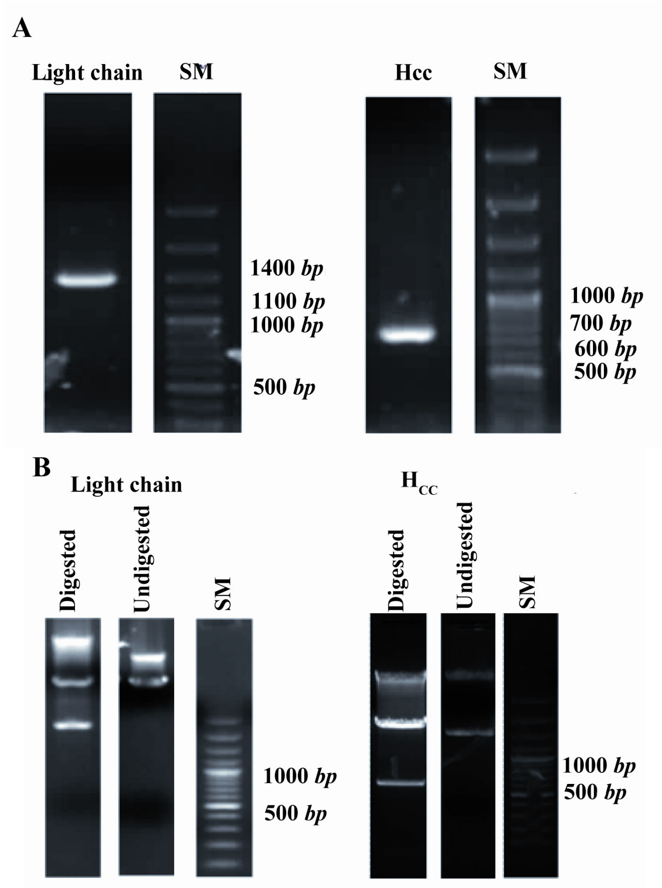


Figure 1. PCR amplification and restriction enzyme digestion of light chain and H_{CC} coding sequences. Agarose gel electrophoresis of PCR products of light chain and H_{CC} fragments confirms their 1371 and 621 *bp* size, respectively; A) Double digestion of pET28b(+) light chain and H_{CC} with BamHI and HindIII endonucleases indicates insertion of these two gene segments into the expression vector; B) SM: DNA size marker, *bp*: base pair

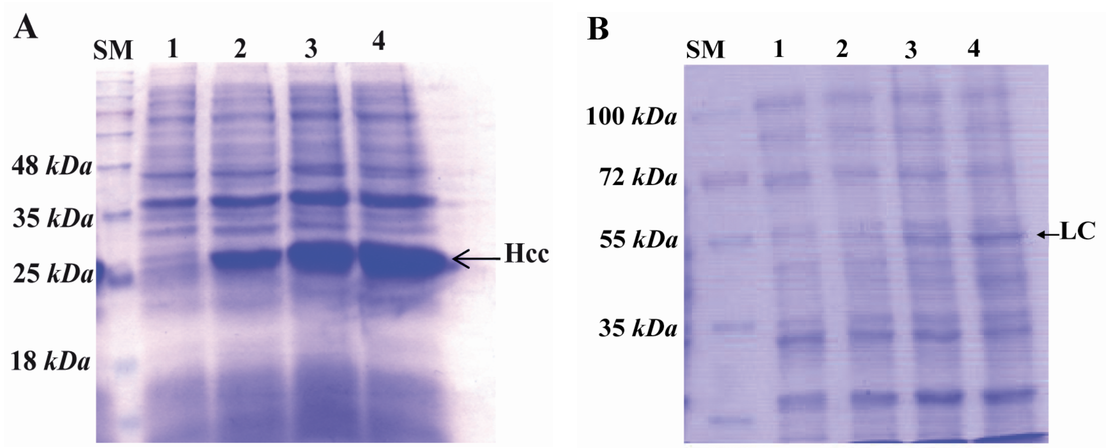
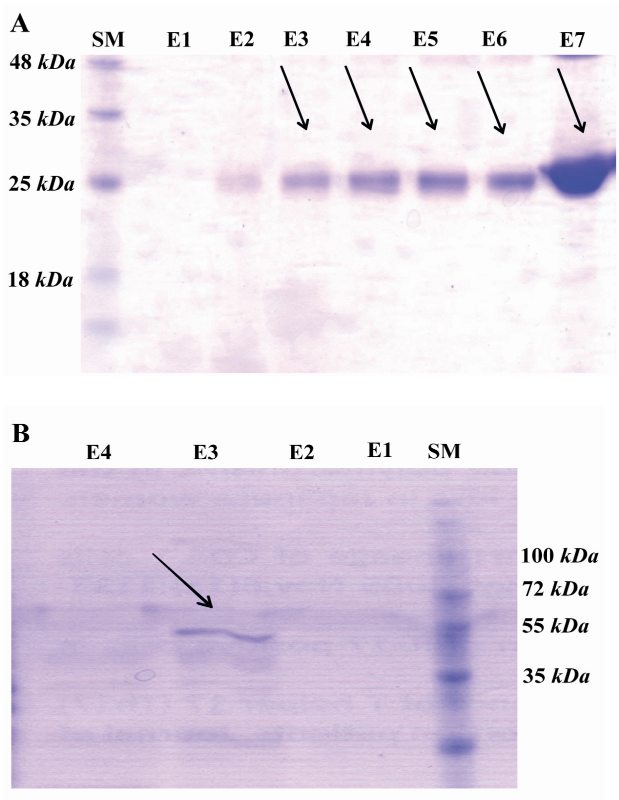


Figure 2. Induction of expression of H_{CC}; A) and light chain; B) proteins in *E. coli* BL21 (DE3). 1 *mM* IPTG was added to a logarithmic liquid culture of transformed bacteria when OD_{600} *nm* was 0.6. Pre-induction (1) and post-induction samples were collected after 2 *hr* (2), 4 *hr* (3) and overnight (4) culture and run on 12% SDS-PAGE followed by Coomassie blue staining. The arrow in the gel shows the expressed protein with the expected molecular weight (~25 and 50 *kDa*, respectively); SM: protein size marker



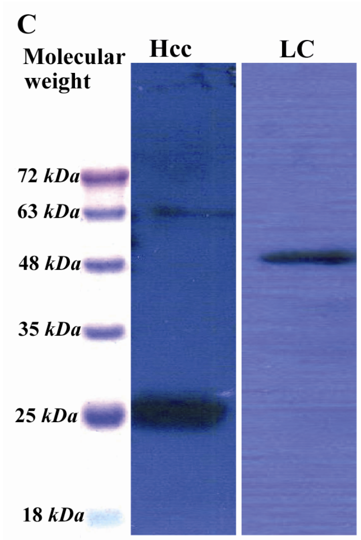


Figure 3. SDS-PAGE electrophoresis; A and B) and immunoblotting; C) profiles of the purified recombinant light chain and H_{CC} proteins. The samples were run on 10-12% polyacrylamide gel and stained with Coomassie blue. Immunoblotting of H_{CC} and LC fragments were performed using human anti-TeNT polyclonal antibodies produced in our lab; C) SM: protein size marker, E1-E7: different fractions of proteins eluted by 500 *mM* imidazole from Ni-NTA column

Table 1. Reactivity of anti-TeNT monoclonal and polyclonal antibodies to tetanus toxin, toxoid, fragment C, recombinant H_{CC} (rH_{CC}) and recombinant light chain (rLC). The results represent OD values obtained at 450 *nm* by ELISA method

mAbs	Toxin	Toxoid	Fragment C	rH_{CC}	rLC
1F3E3	>3.0	>3.0	**1.682**	**1.257**	0.104
1F2C2	>3.0	>3.0	**2.580**	0.066	0.085
1F3B3	0.598	0.915	0.208	0.013	**1.003**
1F3C3	0.687	1.915	0.164	0.074	**0.491**
Human poly anti-TeNT	>3.0	>3.0	1.712	**1.33**	**2.742**
Blank	**0.036**	**0.084**	**0.141**	**0.059**	**0.061**

BL21 (DE3), Tuner and NovaBlue to optimize the expression conditions.

Structural characterization of the recombinant proteins

Ni-NTA purified proteins were checked by SDS-PAGE (Figures 3A and B). Eluted fractions of both H_{CC} and LC proteins were almost devoid of contaminating proteins. To assess the identity and conformation of the purified proteins, immunoblotting and ELISA assays were performed using anti TeNT specific polyclonal and monoclonal antibodies. Our results demonstrated specific reactivity of recombinant H_{CC} and LC with both polyclonal and monoclonal antibodies in immunoblotting (Figure 3C) and ELISA (Table 1) methods.

Discussion

Clostridial neurotoxins belong to classical A-B type toxins by their principal mode of action including an enzymatically active component, "A" and cell binding component "B" [15,16].

Although molecular mechanism of TeNT toxicity is well characterized, the mechanism whereby TeNT binds to neurons requires more investigations. Several lines of evidence indicate that TeNT binding to its receptor depends on gangliosides (notably gangliosides of the 1 b series), and GPI-anchored glycoproteins [17-21]. This gave direct support for involvement of a dual receptor mechanism in the binding of the TeNT in which gangliosides and glycosylated proteins such as synaptic vesicle proteins SV2A and SV2B are involved in TeNT binding. These components are present in both toxin-sensitive PC12 cells and spinal cord neurons [22]. In this regards, application of recombinant DNA technology to produce different parts of TeNT could help to get better understanding of TeNT binding properties and neuronal activity.

In the present study, two recombinant fragments of TeNT were produced and purified. These two proteins play pivotal roles in intoxication and binding of TeNT to neuronal cells. LC cleaves the neuronal SNARE protein and blocks the release of inhibitory neurotransmitter which ultimately leads to spastic paralysis and H_{CC} plays a key role in binding of TeNT to target cells [7]. Our results showed that LC and H_{CC} fragments were successfully expressed in (*E. coli*) BL21 (DE3) and efficiently purified by Ni-NTA chromatography. Recombinant H_{CC} protein was expressed at high levels in (*E. coli*) BL21 (DE3) with approximately 25 *kDa* molecular weight (Figure 2A), whereas LC was only produced in very low amounts with approximately 50 *kDa* molecular weight (Figure 2B). These differences between LC and H_{CC} expression may partly be explained by the fact that LC is the toxic part of TeNT and may have toxicity effect on growth of (*E. coli*) BL21(DE3). We proposed expression of LC using other expression vectors or expression systems such as yeast to overcome toxicity of the protein in (*E. coli*). In addition our results demonstrated that anti-TeNT polyclonal and monoclonal antibodies (mAbs) specifically react with recombinant LC and H_{CC} proteins.

Two previously reported [14] anti TeNT light chain mAbs (1F3B3 and 1F3C3) recognized the recombinant LC whereas only one anti fragment C mAb (1F3E3) binds to recombinant H_{CC} (Table 1). The second fragment C-specific mAb (1F2C2) failed to react with the H_{CC} subdomain. This mAb may either recognize a conformational epitope requiring both H_{CC} and H_{CN} subdomains for its expression or an epitope expressed in only H_{CN} subdomain of fragment C. Alternatively, it may recognize a conformational epitope on H_{CC} which might be lost due to denaturation by 8 *M* urea. Although the purified protein was renatured by a gradient of urea during the purification process (see the Materials and Methods), refolding of the protein might have been incomplete.

Conclusion

In conclusion, our results indicated successful cloning, production and structural characterization of LC and H_{CC} subdomains. Investigation of the immunogenicity and immunoprotectivity of these fragments could extend our understanding about their implication for immunoprophylaxis and treatment of tetanus.

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