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Differential endopeptidase activity of different forms of type A botulinum neurotoxin: A unique relationship between the size of the substrate and activity of the enzyme

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ABSTRACT

Botulinum neurotoxins (BoNTs; serotypes A-G) are metalloproteases, which cleave and inactivate cellular proteins essential for neurotransmitter release. In bacterial cultures, BoNTs are secreted as a complex of the neurotoxin and a group of neurotoxin associated proteins (NAPs). Under physiological condition (pH 7.4), this complex is believed to be dissociated to separate the neurotoxin from NAPs. BoNT consists of a 50 kDa light (L) chain (LC or catalytic domain) and a 100 kDa heavy (H) chain (or HC) linked through a disulfide bond and other non-covalent interactions. The cell intoxication involves three major steps; binding, membrane translocation and inhibition of neurotransmitter release. The last step of intoxication, endopeptidase activity, is very unique and specific that can be used for detection of the complex and isolated forms of the toxin. A fluorescent tag-labeled synthetic peptide (SNAPtide) derived from a segment of SNAP-25, an intracellular substrate of BoNT/A, is used to detect and assay the endopeptidase activity of BoNT/A. The detection of the signal is based on the change in the fluorescence energy transfer after selective cleavage of the peptide by the BoNT/A.

In this report, we demonstrate that SNAPtide as a commonly used substrate widely differ in reaction with BoNT/A complex, BoNT/A, and BoNT/A light chain. These findings have implications for assays used in detection, and in screening potential inhibitors.

1. Introduction

Botulinum neurotoxin (BoNT) is a uniquely potent protein synthesized by *Clostridium botulinum, C. baratii and C. butyricum*, (Rosetto et al., 2014; Kukreja and Singh, 2014). It acts on cholinergic nerve endings and blocks the release of acetylcholine causing flaccid paralysis. It is responsible for the disease botulism, which results in flaccid muscle paralysis and leads to death. Botulism can be spread by accidental food poisoning, wound, air, and liquid. For nearly three decades now it is also being used as a therapeutic agent against disorders of uncontrolled peripheral cholinergic nerve activity, such as dystonia (Kukreja and Singh, 2015). In the disease form, it is considered as the most poisonous of all poisons (Lamanna, 1959; Singh, 2000; Kumar et al., 2014). BoNT, an AB type toxin, is a 150 kDa protein composed of two functional domains: a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC), linked through a disulfide bond. The C-terminal of heavy chain specifically target the neuronal cells and facilitate receptor-mediated endocytosis. Whereas, N-terminal (50 kDa) of heavy chain facilitates translocation of light chain to the cytosol inside the neuronal cells. The LC is a zinc-endopeptidase, and is associated with the intracellular activity of blocking acetylcholine release (Sharma and Singh, 2004; Rosetto et al., 2014). In bacterial culture, BoNT/A is produced in three progenitor toxin forms: M, L, and LL forms. The M form consists of neurotoxin (150 kDa) and a nontoxic protein component (140 kDa) which is called nontoxic non-hemagglutinin component, NTNH (Gu et al., 2012) with 12S molecular size (the molecular size of complex forms is expressed as sedimentation equilibrium values) (East and Collins, 1994). The L form has a molecular weight of about 500 kDa and a molecular size of 16S. The LL form is about 900 kDa and 19S. The L and LL complexes consist of several hemagglutinin components besides the BoNT and NBP, and exhibit hemagglutinin activity (Somers and DasGupta 1991; Fujii

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1995; Singh et al., 2014). These are referred to as neurotoxin associated proteins (NAPs), and also as complexing, ancillary, or accessory proteins (Singh et al., 2014). Stabilized through non-covalent interactions, NAPs account for up to 80% of the total mass of BoNT complex. In case of natural botulism, it is the complex form of BoNT that is encountered. In general, BoNT in the complex form is resistant to environmental stress, such as pH, temperature, and proteases. L form of the complex is used in this study.

The different serotypes (A-G) have unique receptors to which they bind to produce the toxic effect on the nerve cells. (Lance, 2004; Rosetto et al., 2014). Intracellularly, the endopeptidase activity of different serotypes of BoNT target selectively a unique group of proteins, the SNARE proteins (Kumar et al., 2014; Rosetto et al., 2014). BoNT/A and BoNT/E cleave SNAP-25 (synaptosomal associated protein), (Blasi et al., 1993; Schiavo et al., 1993; Binz et al., 1994), BoNT/C cleaves both syntaxin and SNAP-25 (Blasi et al., 1993) and BoNT/B, BoNT/D, BoNT/F and BoNT/G cleave VAMP (Niemann et al., 1994). Interestingly, cleavage sites for each of these BoNT serotypes are unique and very specific.

It becomes necessary to detect the presence of this deadly bacterial toxin since it is capable of contaminating air, liquid and solid food. If fallen into the wrong hands BoNT could lead to bio-terrorism and fatal casualties (Franz et al., 1997; Altas, 2002). Appropriate biological assays are needed to detect, diagnose, and develop antidotes.

There are a few assay platforms which have been developed for detection of botulinum neurotoxin. Mouse bioassay is the gold standard and most sensitive technique for the detection of biologically active BoNT, but there are several drawbacks (Cherington, 2004; Kukreja and Singh, 2014). This technique is expensive to perform, requires sacrificing of mice and is not specific for a BoNT serotype unless used in combination with antibodies. Moreover, it takes up to 4 days to complete, which makes it unsuitable for rapid detection and screening. Because of its disadvantages, various attempts are being made in order to replace the mouse bioassay. Another technique used for the detection of botulinum neurotoxins is ELISA, but this technique also is time-consuming. One of the other disadvantages of ELISA is that it is able to detect the presence of BoNT but it fails to distinguish if the toxin is in its active or inactive state (Moorthy et al., 2004). Therefore, it is important to have an assay that is cost-effective, easy to perform, robust, easy to handle and has high sensitivity.

The cleavage of the BoNT endopeptidase substrate or its derived peptide (e.g., SNAPtide) is commonly used for assaying and detection (Wang and Singh, 2010, 2012; Feltrup and Singh, 2012). Peptide substrates of varying length (15–66 residues) have been tested for efficient cleavage and kinetics, with varying results (Schmidt and Bostian, 1995; Silhar et al., 2010). List Biologicals developed a 13-mer peptide by replacing the Glutamine-194 of SNAP-25 with glutamic acid SNAPtide, which showed catalytic efficiency (Kcat/Km) of LCA as $28.5 \times 10^4\ M^{-1}sec^{-1}$ (Todd et al., 2005), which is comparable to full-length substrate GST-SNAP25 is $27.0 \times 10^4\ M^{-1}\ s^{-1}$ (Baldwin et al., 2004). Additionally, Breidenbach and Brunger (2004), demonstrated the involvement of α and β exocites in the endopeptidase activity of BoNT/A LC. These exocites are involved in the binding and recognition of the substrate. Therefore, the requirement of a longer peptide as a substrate is unique for the BoNT endopeptidase. It is also known that the size of the enzyme and substrate make a difference in the enzyme activity (Wang and Singh, 2010; Mizanur et al., 2013). The requirement of longer substrate raises the following questions: a) Does the size of enzyme and substrate play a role in the endopeptidase activity and kinetics of proteolysis? and b) What is the role of different domains and associated proteins in the endopeptidase activity of this enzyme? Additionally, the uniqueness of active site (20 Å deep negatively charged active site), the presence of exosites (α and β), and unique substrate recognition mechanism make this problem more interesting (Breidenbach and Brunger, 2004). In other words, detection of toxin is not just an analytical problem, it needs a careful consideration of the biochemical function of this molecule.

Given the fact that there are at least three forms (sizes) of the endopeptidase (L chain, BoNT toxin consisting of L and H chains, and BoNT in the complex form) that are encountered under natural conditions, a systematic comparative enzyme assay study is needed for each of the BoNT serotypes.

In this study, we have examined endopeptidase activity of BoNT/A complex, BoNT/A toxin and BoNT/A LC against the fluorescent tagged peptide substrate (derived from its substrate, SNAP-25) using the Forster Resonance Energy Transfer (FRET) technique, and against a full-length SNAP-25 substrate. The results indicate a drastic difference in the enzyme activities of the three forms of the endopeptidase against the peptide substrate, which vary from the activities with the full-length substrate.

2. Materials and methods

2.1. Materials

SNAPtide®520 (oAbz/Dnp) was purchased from List Biologicals (Campbell, California, U.S. Patent #6,504,006). It is a polypeptide consisting of 13 amino acids having 2, 4-Dinitrophenol at its C-terminal and Orthoamino benzoic acid at its N-terminus (Fig. 1) and possesses a cleavage site for BoNT/A. Distilled water was used in preparing all the buffers. Full-length substrate, His-SNAG (6XHis-SNAP-25-EGFP) was purified according to the procedure described below.

2.2. Isolation of BoNT/A complex BoNT/A toxin and BoNT/A L chain

BoNT/A complex, BoNT/A toxin and BoNT/A L chain were isolated according to the previously published procedures (Li and Singh, 1999; Singh et al., 2014). BoNT/A complex and BoNT/A toxin were stored at −80 °C in 20% glycerol until use. The samples were thawed and passed through Sephadex ™ G-25 (1 × 7 cm) by Amersham Biosciences and eluted with the cleavage buffer [50 mM Tris base, 10 mM $NaH_2PO_4 \cdot H_2O$, 300 mM NaCl, 0.3 mM $CaCl_2$, 30 mM NaN_3, 2 mM $MgCl_2 \cdot 6H_2O$ and 1 mM mercaptoethanol (pH 7.6)]. For nonreducing conditions, the cleavage buffer did not

Fig. 1. The sequence of the SNAPtide and SNAP-25. The Q (Glutamine) from SNAP-25 is replaced by E (glutamic acid) at position 194 in the SNAPtide. The BoNT/A cleaves between (Gln)Q^{197} and (Arg) R^{198}.

contain mercaptoethanol. The BoNT/A L chain was freshly isolated and dialyzed against the buffer. For the nonreducing conditions, the BoNT/A was dialyzed against cleavage buffer minus mercaptoethanol. 10 mM Sodium Phosphate, pH 7.4, containing 300 mM NaCl and 2.5 mM mercaptoethanol was used as a cleavage buffer for all the endopeptidase reaction.

2.3. Preparation of full length SNAP-25

E coli BL21 DE3 cells were transformed with the plasmid of His-SNAG (His-SNAP-25-EGFP) clone. Transformed cells were plated in carbenecilline plate, and a single colony was selected for overnight growth at 37 °C. Cells were added to 1 lt flask and grown at 37 °C. Cells were induced at OD 0.6 to 0.8 by adding 1 mM IPTG for 15 h at 25 °C. The cell pellet was harvested by centrifuging the media, which was lysed by sonication (in equilibration buffer; 10 mM sodium phosphate, pH 7.4 containing 300 mM NaCl with protease inhibitor cocktail (Roche, IN) and 2 mM PMSF). The supernatant after centrifugation was loaded onto a pre-equilibrated Ni-NTA column. After washing with equilibration buffer (10 mM sodium phosphate, pH 7.4, containing 300 mM NaCl and 10% glycerol) and wash buffer (10 mM sodium phosphate, pH 7.4, containing 300 mM NaCl, 10% glycerol, containing 20 mM imidazole), bound protein was eluted with elution buffer (10 mM sodium phosphate, pH 7.4, containing 300 mM NaCl, 10% glycerol, and 100 mM Imidazole). The pooled pure fractions were dialyzed in 10 mM sodium phosphate, pH 7.4, containing 300 mM NaCl and 20% glycerol, and stored at −80 °C.

2.4. Assay with the peptide substrate

BoNT/A dissolved in cleavage buffer [50 mM Tris base, 10 mM $NaH_2PO_4 \cdot H_2O$, 300 mM NaCl, 0.3 mM $CaCl_2$, 30 mM NaN_3, 2 mM $MgCl_2$ and 1 mM mercaptoethanol (pH 7.4)] to a final concentration of 100 nM, was incubated at 37 °C for 30 min before adding it to the reaction mixture (700 μl). For non-reducing conditions BoNT/A was dissolved in the cleavage buffer, not containing mercaptoethanol, to a final concentration of 100 nM per reaction (700 μl).

Cleavage of SNAPtide ceases FRET between donor and acceptor, thus increasing the intensity of the fluorescence that was measured by the fluorimeter in the steady-state mode. The reaction mixture consisted of 15 μM SNAPtide, 100 nM of BoNT/A L chain or BoNT/A complex and BoNT/A toxin in the reduced and non-reduced conditions. The excitation wavelength was 320 nm and the emission spectra were recorded between 340 nm and 460 nm, using the K2 spectrophotometer (ISS Inc., Urbana, IL). The excitation and emission slit widths were fixed at 16 nm and 4 nm respectively. The highest emission peak was observed at 418 nm. The emission was recorded every 5 min for an hour at 37 °C to monitor the reaction progress. All the experiments were done in parallel.

2.5. Endopeptidase activity with His-SNAG

The endopeptidase activity of the enzyme was determined by using full-length His-SNAG-substrate which gave two cleavage products 28 kDa and 24 kDa band, as determined from the SDS-PAGE. For the quantitative determination of activity, densitometric analysis was performed on the intensity of full length substrate band, at each time point. Prior to endopeptidase reaction, required concentration of the enzyme was incubated in reaction buffer (1x PBS, pH 7.4, containing 1 mM DTT) at 37 °C for 30 min. The concentration of the enzyme used was 30 nM. The substrate (SNAG) concentration was 4 μM, and the reaction was performed at 37 °C. The reaction was stopped by adding SDS-PAGE running buffer to the reaction mixture, followed by boiling the sample for 3 min in a water bath. The reaction results were examined and viewed by running the precast mini SDS-PAGE (4–20% Tris.HCl, 10 wells, Bio-Rad Laboratories). The densitometric analysis of the inhibitor was performed using the Bio-Rad Image lab 5.2.1 software. Results reported are representative of at least two replicates. All the experiments were done in parallel.

3. Results

Assay of botulinum toxin activity is critical for the risk-assessment, diagnosis, and treatment of botulism cases. The frequently encountered active forms of the toxin are; L chain (inside intoxicated neuronal cells), BoNT (in the blood stream of a botulism patient, and in the form of a complex (in food samples, gastric juices, and intestinal aspirate). It has already been reported that there is a difference between the endopeptidase activity of the BoNT/A toxin and BoNT/A complex, former showing optimal endopeptidase activity only under the conditions when the disulfide bond between the L and H chain is reduced, whereas BoNT/A complex showing nearly full endopeptidase activity without the reduction of the disulfide bond (Cai et al., 1999). In other words, the botulinum toxin complex is fully active without reduction. An intriguing question arises, why there is no need for the reduction of the disulfide bond in the complex? Do associated proteins help toxin get the right conformational state for nearly optimal endopeptidase activity? A toxin detection platform based on the endopeptidase activity needs characterization of the endopeptidase activity of the different samples, using a common substrate. Similarly, development of antidotes against the endopeptidase activity requires assay of the enzyme activity of all the forms of botulinum toxin.

The present research deals with the investigation of the activity of different forms of BoNT/A in reduced and non-reduced conditions. The reaction was carried out at 37 °C for 1 h and their activities against a FRET peptide (SNAPtide) were observed with excitation and emission at 320 nm and 418 nm, respectively. The enzymatic activity of BoNT/A in reduced form with the full-length substrate (His-SNAG) is examined to compare the effect of substrate size on the enzymatic activity of the three forms of the toxin.

3.1. Endopeptidase activity of complex

The endopeptidase activity was assayed using peptide as well as full-length SNAP-25 substrates. BoNT/A complex was assayed both in reduced and non-reduced conditions (Fig. 2). In the peptide substrate assay measuring the fluorescence, increase upon cleavage by the enzyme, the activity remains linear for the first 20 min, and then it becomes biphasic (Fig. 2b). Since the activity remains most linear for the first 20 min, the rate of the cleavage activity of SNAPtide by the complex was calculated during this period (Table 1). Within these first 20 min 52% SNAPtide was cleaved by the complex in the reduced state, whereas only 20% was cleaved by the complex in the non-reduced state, showing very little activity of the complex in the non-reduced state. The relative substrate cleavage of reduced and non-reduced BoNT/A complexes are listed in Table 1 in which the former shows 2.6-fold higher cleavage than the latter. In order to analyze its initial rate of activity, the percentage cleavage was calculated for the first 5 min of the reaction, and it was observed that complex in the reduced condition cleaved 37% of SNAPtide and only 16% in its non-reduced state.

The time course of His-SNAG cleavage was monitored by BoNT/A Complex under reducing conditions. Prior to the reaction, BoNT/A complex was incubated for 30 min at 37 °C, followed by incubation at the same temperature the substrate-enzyme mixture for up to 60 min. The reaction was terminated, at each time point, by adding

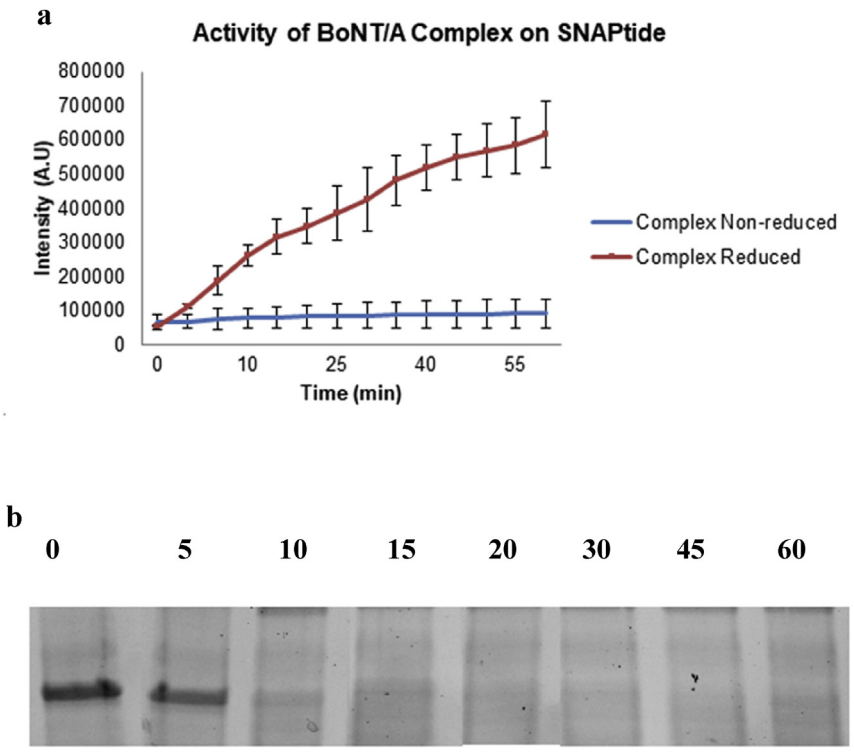

Fig. 2. **a**: Comparison of the cleavage activity of the BoNT/A complex in reduced and non-reduced conditions continuously monitored every 5 min at 37 °C. The reduced state of the BoNT/A complex showing more activity than its non-reduced state. The reduced state shows a biphasic system. Each set of data was performed in triplicates and the mean of triplicates were taken for the data reported for SNAPtide assay. Statistical analysis was done using EXCEL. Data recorded at 0 min is the Control containing only SNAPtide. **b**: The time course of His-SNAG cleavage by BoNT/A complex under reducing conditions. Prior to reaction, BoNT/A LC was incubated for 30 min at 37 °C in 10 mM Sodium Phosphate (Na-P), pH 7.4 containing 300 mM NaCl and 1 mM Dithiothreitol (DTT). His-SNAG concentration was 4 μM. Both substrate and enzyme were incubated for 0, 5, 10, 20, 30 and 60 min (indicated by numbers above the gel). Reaction was then terminated, at each time point, by adding SDS-sample buffer. Samples were then separated on a 4–20% gel (Bio-Rad) and stained with Coomassie blue staining. Intensity of uncleaved substrate was analyzed by densitometric analysis. Results reported are representative of at least two replicates. Data recorded at 0 min is the control containing only His-SNAG. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
The percentage cleavage activity of BoNT/A Complex, BoNT/A and BoNT/A L chain in reduced (R)and non-reduced (NR) states with SNAPtide. The graphs (Figs. 2a, 3a and 4a) obtained were most linear in the first 20 min hence the percentage was calculated within the first 20 min, recorded at 0, 5, 10, 15 and 20 min. The average intensity values were used for the percentage cleavage activity.

Time, min	BoNT/A Complex R	BoNT/A Complex NR	BoNT/A Toxin R	BoNT/A Toxin NR	BoNT/A LC R	BoNT/A LC NR
00	22.2	8.9	22.8	22.8	68.4	73.4
05	37.0	16.0	61.0	30.9	83.3	81.9
10	50.0	15.2	79.4	39.4	85.9	86.5
15	50.0	18.6	82.1	47.2	87.7	87.3
20	51.7	20.8	84.5	53.5	88.0	90.0

SDS-PAGE sample buffer. As shown in the SDS-PAGE gel (Figs. 2b, 6), the intensity of the band reduces with time showing the cleavage activity. The percentage of cleavage was found to be 92.3% (Table 2) in the first 20 min showing that the complex was comparatively more active in its reduced state with His-SNAG than SNAPtide. BoNT/A complex in the reduced state was found to be more active than the BoNT/A complex in the non-reduced state. The activity of the BoNT/A complex in the reduced and non-reduced states were substantially different (over 2-fold; Table 1), with BoNT/A complex under reduced conditions showing higher activity than under non-reducing conditions.

Table 2
Percentage cleavage activity of Complex A, Toxin A and BoNT/A LC in reduced states with full length His-SNAG.

Time, min	Complex	BoNT/A toxin	BoNT/A LC
00	0	0	0
5	64.6	29.2	7.0
10	82.9	39.0	22.4
15	87.5	46.5	30.5
20	92.3	50.8	37.0

3.2. Endopeptidase activity of BoNT/A toxin

The percentage cleavage within the first 20 min of BoNT/A toxin in the non-reduced state was found to be only 54% which was significantly less compared to the reduced state of the BoNT/A toxin, 85% (Table 1). The enzymatic activity was similar soon after the toxin was mixed with SNAPtide for both reduced and non-reduced conditions, but had increased activity in the first 5 min in the reduced condition, cleaving almost 61% of the peptide substrate, SNAPtide, as compared to the non-reduced state where it

cleaved only 31%.

For full-length substrate, the toxin was incubated for 30 min at 37 °C before adding it to the reaction mixture. The reaction mixture was incubated for up to 60 min at 37 °C. The reaction was stopped by adding SDS-sample buffer at 0,10, 20, 30 and 60 min. The SDS-PAGE gel (Fig. 3b, Fig. 6), reveals the cleavage activity of the toxin as we see the His-SNAG band fades with incubation time. Only 50% of the substrate was cleaved in the first 20 min of the reaction (Table 2).

BoNT/A toxin exists in a form where the LC is attached to the HC by the disulfide bond. Once the bond is broken in the reduced condition, the toxin shows a higher activity than in the non-reduced state (Fig. 3a). Compared to the BoNT/A complex, under both conditions (reduced and non-reduced), BoNT/A toxin is comparatively more active with the peptide substrate. However, with full-length substrate, the BoNT/A complex cleaves faster compared to the isolated toxin.

3.3. Endopeptidase activity of BoNT/A LC

Initial activity (in first 20 min) of reduced and non-reduced BoNT/A LC with peptide substrate was 90% and 88% (Fig. 4a; Table 1), respectively. The reaction was instantaneous and shows 82% of the SNAPtide was cleaved during the first 5 min (Fig. 5). The fluorescence intensity in the BoNT/A LC cleavage under the reduced state increases with time and eventually becomes more than the non-reduced state of BoNT/A LC (Fig. 4). In the FRET analysis, the cleavage activity of the BoNT/A LC in the reduced state and non-reduced state was found to be approximately the same. A notable point was an instant increase in the cleavage upon mixing of the BoNT/A LC and the SNAPtide substrate, both under the reducing and non-reducing conditions.

Under the condition where the full-length substrate was used with BoNT/A LC, the LC was incubated for 30 min at 37 °C before adding it to the reaction mixture and the action was stopped by adding SDS-PAGE sample buffer. The SDS-PAGE gel shows very little activity (Fig. 4b). The activity of BoNT/A LC with full-length substrate showed only a small change and significant amount of uncleaved substrate was still present after 60 min of the reaction (Figs. 4b, 6). The BoNT/A LC cleaved only 37% of His-SNAG in the first 20 min of its reaction, and only 22% in the first 5 min of the reaction (Table 2).

The results also suggest that the BoNT/A toxin and BoNT/A complex are more active when the disulfide bond is reduced. This suggests that the reduction of the disulfide bond is necessary for the optimum activity of the botulinum toxin as previously suggested (Cai et al., 1999). The BoNT/A toxin with the full-length substrate is only slightly less active than with the SNAPtide.

4. Discussion

A large-scale assay for active botulinum neurotoxins is essential in a variety of conditions. These include screening of antidote candidates, diagnostics of botulism, detection of botulinum neurotoxins in food samples, environment, and testing of the activity of botulinum neurotoxin therapeutics for batch releases. The samples for these conditions range from light chain of the toxin to its complex form consisting of BoNT toxin and NAPs. Being the most

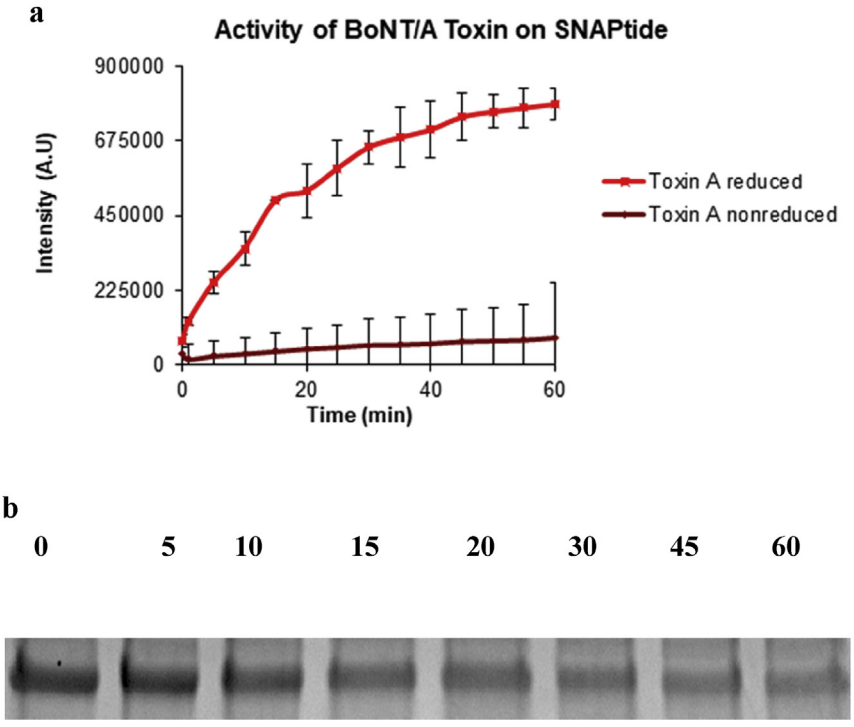

Fig. 3. a: FRET analysis of the cleavage activity of the BoNT/A toxin in reduced and non-reduced conditions continuously monitored every 5 min at 37 °C, with the reduced state of the BoNT/A showing more activity than its non-reduced state. Each set of data was performed in triplicates and the mean of triplicates were taken for the data reported for SNAPtide assay. Statistical analysis was done using EXCEL. Data recorded at 0 min is the Control containing only SNAPtide. **b**: The time course of His-SNAG cleavage by pure BoNT/A toxin under reducing conditions. Prior to reaction, BoNT/A LC was incubated for 30 min at 37 °C in 10 mM sodium phosphate, pH 7.4 containing 300 mM NaCl and 1 mM DTT. His-SNAG concentration was 4 μM. Both substrate and enzyme were incubated for 0, 5, 10, 20, 30 and 60 min (indicated by numbers above the gel). Reaction was then terminated, at each time point, by adding SDS-sample buffer. Samples were then separated on a 4–20% gel (Bio-Rad) and stained with Coomassie blue staining. Intensity of uncleaved substrate was analyzed by densitometric analysis. Results reported are representative of at least two replicates. Data recorded at 0 min is the control containing only His-SNAG. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

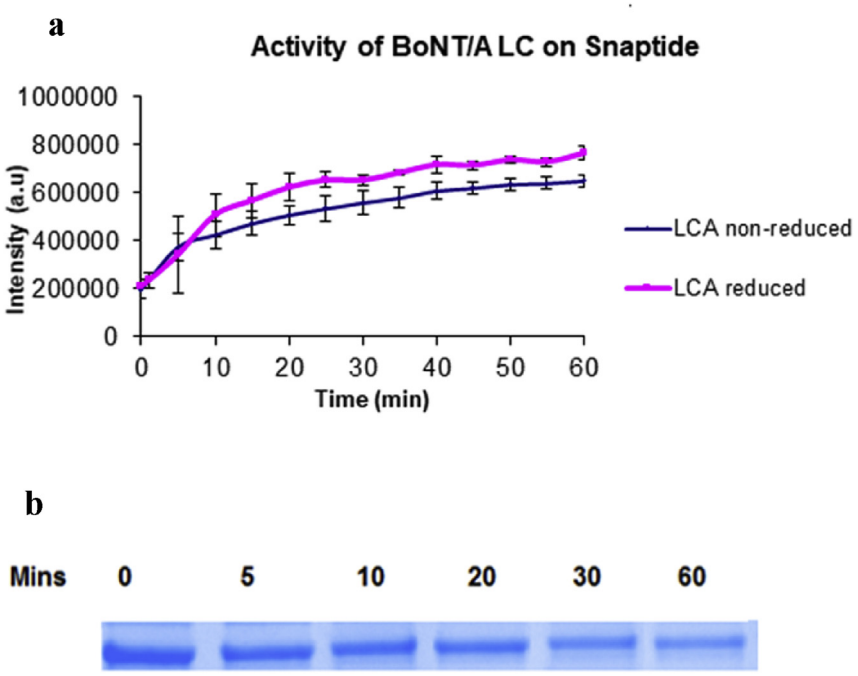

Fig. 4. **a**: Comparison of the cleavage activity of the BoNT/A LC in reduced and non-reduced conditions continuously monitored every 5 min at 37 °C, having almost the same endopeptidase activity. Each set of data was performed in triplicates and the mean of triplicates were taken for the data reported for SNAPtide assay. Statistical analysis was done using EXCEL. Data recorded at 0 min is the Control containing only SNAPtide. **b**: The time course of His-SNAG cleavage by pure BoNT/A LC under reducing conditions. Prior to reaction, BoNT/A LC was incubated for 30 min at 37 °C in 10 mM Na-P, pH 7.4 containing 300 mM NaCl and 1 mM DTT. His-SNAG concentration was 4 μM. Both substrate and enzyme were incubated for 0, 5, 10, 20, 30 and 60 min (indicated by numbers above the gel). Reaction was then terminated, at each time point, by adding SDS-sample buffer. Samples were then separated on a 4–20% gel (Bio-Rad) and stained with Coomassie blue staining. Intensity of uncleaved substrate was analyzed by densitometric analysis. At each time points, three separate experiments were carried out. Data presented in the figure is the average of the three experiments with standard deviation. Data recorded at 0 min is the control containing only His-SNAG. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

versatile, BoNT/A is commonly encountered form of BoNT intoxication. Peptide (SNAPtide) based fluorescence assay is very convenient and commonly used method for measuring BoNT/A activity. However, it is important that assay for each of these samples is examined for a given substrate.

Amongst the three forms of type A botulinum neurotoxin endopeptidase tested against SNAPtide as the substrate, BoNT/A complex was found to be the least active showing minimum endopeptidase activity. The BoNT/A LC, on the other hand, showed the highest activity. The BoNT/A complex is seen to be the most active when full-length HIS-SNAG was used as a substrate.

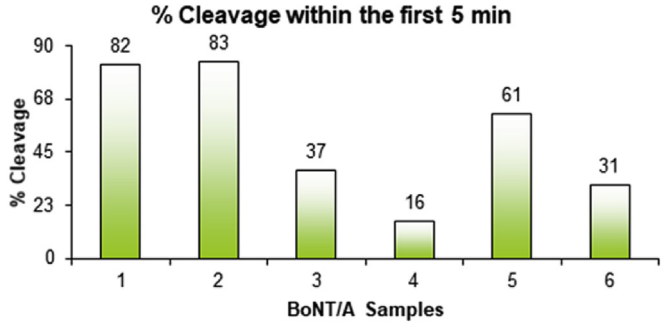

Fig. 5. The percent cleavage activity of BoNT/A toxin, BoNT/A complex and BoNT/A LC in its reduced and non-reduced states. The fluorescence intensity was observed at 418 nm and the average value was calculated to determine the percentage cleavage. The graphs obtained were most linear in the first 5 min hence the percentage was calculated within this period. At each time points, three separate experiments were carried out. Data presented in the figure is the average of the three experiments with standard deviation. Lane 1 is non-reduced BoNT/A LC, lane 2 is reduced BoNT/A LC, lane 3 is reduced BoNT/A Complex, lane 4 is non-reduced BoNT/A Complex, lane 5 is reduced BoNT/A Toxin and lane 6is non reduced BoNT/A Toxin.

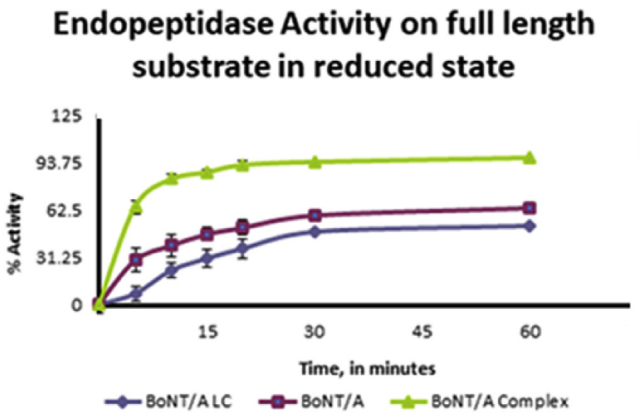

Fig. 6. The endopeptidase activity of BoNT/A in the reduced state was determined and the cleavage activity was measured to be the highest in the presence of full length substrate (His-SNAG). At each time points, three separate experiments were carried out. Data presented in the figure is the average of the three experiments with standard deviation. Data recorded at 0 min is the control containing only His-SNAG.

The results demonstrate that the reduction of the disulfide bond is one of the requirements for getting better endopeptidase activity as the nonreduced states show lesser activity. BoNT/A LC is equally active in reduced and non-reduced conditions in the first 20 min with SNAPtide as the substrate due to the absence of the disulfide bond. BoNT/A LC in the reduced state with SNAPtide (88%) as the substrate is observed to have similar activity as the BoNT/A complex with full-length His-SNAG (83%) substrate. (Tables 1 and 2). Since the SNAPtide consists of a cleavage site along with short

segments of neighboring peptide residues, the LC cleaves the SNAPtide almost instantly, and we observed the rise in fluorescence reporter signal. But endopeptidase activity under natural conditions involves three different events; a) recognition and binding of the substrate, which involves domains other than the peptide segment containing the cleavage site of the substrate and the enzyme active site, b) exposure of active site as a result of substrate binding, and c) cleavage of the substrate. Peptide substrate lacks recognition and binding to distal sites, its relatively small size allows it to reach the enzyme active site for specific binding and cleavage. The fact that the peptide substrate (SNAPtide) exhibits even slightly higher enzymatic cleavage than the full-length SNAP-25 with light chain implies that in the absence of the heavy chain it has higher accessibility to the active site. This would imply that the additional binding of the substrate to exosites in the BoNT/A toxin pushes the active site out from the obfuscation of the heavy chain. Furthermore, the presence of NAPs apparently makes the active site less accessible to the peptide substrate, as shown by the significantly lower endopeptidase activity of BoNT/A complex with the peptide substrate compared to even BoNT/A toxin (Tables 1 and 2). Interestingly, the full-length substrate exhibits the highest activity with the complex and lowest with the light chain. These observations suggest that there may be synergistic effects of the substrate binding to exosites in the presence of either heavy chain and/or more so NAPs. Further studies are needed to understand this unique and novel observation. Notably, the full-length SNAP-25 is tagged with GFP. Although GFP tag is not in the vicinity, of the binding site of the substrate or α & β exosites, it is possible that GFP tag can impede binding and recognition of the substrate. These observations should be confirmed with the SNAP-25 substrate without any tag to better mimic the in vivo conditions.

A comparative analysis of the cleavage activity of different forms of the BoNT/A endopeptidase in reduced conditions with SNAPtide and full-length SNAP-25 substrate is summarized in Figs. 5 and 6. The BoNT/A LC was found to be the most active against SNAPtide, a small peptide segment of the SNAP-25 whereas the BoNT/A complex was the most active against the full-length substrate. The peptide substrate interacts with the active site of BoNT/A LC and cleavage is almost instantaneous due to its small size, although it may not be optimal. The BoNT/A complex, on the other hand, showed the highest activity with the full-length substrate. It has been observed that the BoNT/A endopeptidase (LC), as well as BoNT/A, toxin exist in a molten globule state in the reduced condition at 37 °C (Cai and Singh, 2001; Kukreja and Singh, 2005). The flexible structure of molten globule conformation in BoNT/A toxin allows the enzyme to adapt to a variety of different conditions and modify its secondary and tertiary structure to accommodate its substrate, while retaining its endopeptidase properties due to the native-like folding pattern at physiological temperatures. The above explanation seems to be fitting with the requirement of the large substrate for optimal activity. To accommodate such a large substrate not only active site should be flexible, it also requires other interacting regions of the enzyme (exosites) to have sufficient flexibility (in this case whole enzyme because the substrate encircles the whole enzyme). Also, the binding could result in structural changes leading to exposure of the active site, hence enhancing the enzymatic activity. BoNT/A complex was found to be the least active among the rest with SNAPtide. The neurotoxin associated proteins (NAPs) either obstruct the active site or alter the folding of the endopeptidase domain leading to inaccessibility of the small peptide substrate. Since SNAPtide contains only the cleavage site with some neighboring residues, there is a possibility that the lack of binding sites other than the active site results in failure of the exposure of the active site to be as enzymatically active.

The BoNT/A complex in the reduced state against the full-length substrate demonstrates the similar behavior, suggesting the importance of the steps involved in the endopeptidase activity of the BoNT/A. The bigger molecule behaves better in the presence of a bigger substrate similarly smaller molecule works best with a smaller substrate. This observation can be hypothesized as follows; larger the enzyme larger the substrate required for the optimal activity, and smaller the enzyme smaller the substrate required for the optimal activity (explained in detail in the above paragraph). The SNAPtide does not have the exosite binding, and in turn not able to expose the active site of the BoNT/A complex for the efficient enzymatic activity. Moreover, the heavy chain may be occluding the active site as well, as it is well known that the belt region of the heavy chain binds to the same region of the endopeptidase active site where the substrate binds (Barbieri et al., 2007). The results also suggest that the length of the substrate plays a vital role in its enzymatic activity.

There are various inherent challenges associated with the detection of toxins, such as matrix, sampling procedure, concentration, interference, and sensitivity of the method. An ideal detection system should include high specificity and sensitivity, fast response time, capability for mass production, elimination or simplification of the sample preparation steps, minimal perturbation of the sample, and availability of continuous data analysis. Detection of botulinum toxins has some additional requirements such as speed, safety, handling, and regulatory compliance. The present work can address some of these issues. There are three types of situations for BoNT detection. (A) Patients of botulism outbreaks whose blood, gastric aspirates, or stools are examined for the presence of BoNT. (B) Bioterrorism or intentional spread or contamination of the environment, water, milk, or food supply. (C) Biothreat to defense forces in the military through aerosol exposure. Detection requirements for these situations are different in terms of sensitivity, identification, active vs. inactive toxins, and speed of detection. Sample conditions are also different in these situations. For example, in the situation (A) high sensitivity is critical to diagnose botulism. In situations (B) and (C) sensitivity may not be as critical as it is likely that higher amount of toxin will be involved, but it is important to know if the toxin is active to avoid false alarm and response, including a military response. The experiments conducted in this study will be very useful in these situations (situation (B) and (C) above).

In addition, an important issue related to this molecule is designing an effective antidote/inhibitor. A critical step in developing effective antidotes against any molecule requires a comprehensive knowledge of its active conformational states in solution, and the molecular basis of its function (kinetics and interaction). In general, a crystal structure can address this issue (to a certain extent). However, a crystal structure is largely a static structure, and protein molecules involve several dynamical features in solution to execute their functions. The crystal environment undermines these aspects. In the case of full-length BoNT/A LC, solubility issues and flexibility of the C-terminus have proven difficult to overcome for crystallization despite multiple efforts by researchers to solve the crystal structure. Another factor is the potency of the molecule, and in case of BoNT this factor plays a huge role. High potency means a very delicate play between flexibility, folding, and molecular functions. In other words, to achieve a high potency, the molecule needs to be flexible to adapt its structure in different environments to be more effective, stable, or able to avoid changes in its active form due to the hostile environment, thus, lasting for a longer time without losing its function. Kukreja and Singh (2005) and Kumar et al. (2014) support this observation, which strengthens the hypothesis that this molecule has a different representation in solution than what is known from the published crystal structures. The fact that different forms of the toxin respond to different sizes of

the substrate strongly supports that there is a clear role of folding and flexibility in recognition and biological function of the BoNT. These two parameters need to be taken into consideration while designing an inhibitor for this molecule targeting the endopeptidase activity in different forms of the enzyme encountered under natural conditions.

Finally, because of high potency and potential use of biothreat agent, detection of active toxin in the environmental, food and clinical sample is imperative. For sensitive detection technology, we need to have a better understanding of BoNT biological activity. The findings of this research provide us the insight into the potentially rhythmic movement of the enzyme against the substrate, as evidenced by the size effects of enzyme and substrates. Carefully designed experiments on this aspect will unravel this unique enzyme-substrate interaction, which can further help in designing antidote for this molecule, and better detection technology.

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