

Research paper

Development of improved SNAP25 endopeptidase immuno-assays for botulinum type A and E toxins

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Abstract

Botulinum neurotoxins contain proteases that cleave specific intra-neural proteins essential for neurotransmitter release. Toxin types A, E and C₁ intra-cellularly cleave SNAP25 resulting in a flaccid paralysis. As a consequence, various different endopeptidase assays have been developed to specifically detect the toxins enzymatic activity, however, many of these suffer from variability, low sensitivity or unwanted interference exerted by product specific excipients. The current studies utilised solid phase synthesized SNAP25_{137–206} peptide substrate, and specific antibody to either the SNAP25_{190–197} or _{173–180} octapeptide epitopes that become exposed following cleavage by toxin types A or E respectively. Assay sensitivity was increased 50 fold by the use of an optimal 0.5% Tween 20 concentration in tandem to 0.1% albumin together with an improved, simplified assay design without a pre-activation / reduction step. Sensitivities capable of detecting 0.01 LD₅₀/ml (40fg/ml or 0.3fM) of type A toxin was achieved with a linear dose response between 0.1 and 1 LD₅₀/ml. This provides sufficient sensitivity and precision (inter assay GCV of < 2%) for monitoring activity within any current or newly marketed therapeutic products containing less units per vial and may also make it applicable for other applications. Both purified haemagglutinin free and complexed toxins could be detected equally. Unlike type A, type E activity could unexpectedly be detected in the complete absence of reducing conditions and the optimal assay had a limit of detection of 0.2LD₅₀/ml (4.8pg/ml) with a linear dose response between 1 and 10LD₅₀/ml. The principle of using a detecting antibody to a substrate sequence buried within the native substrates alpha-helix may be further expanded to other specific enzyme cleavage reactions in the future.

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1. Introduction

Seven types or serotypes (A to G) of botulinum toxin are currently known. Each serotype is composed of a heavy and a light chain linked together by disulphide

bonds. The N-terminal end of the heavy chain is responsible for binding to specific pre-synaptic neuronal cell receptors (Synaptic Vesicle protein 2 and gangliosides, for type A toxin) and the C-terminal end facilitates internalisation (Montecucco et al., 1994; Dong et al., 2006). Once inside the cell, the light chain transverse the membrane of the endocytotic vesicles and cleaves specific proteins within the cytoplasm essential for the docking and fusion of neurotransmitter containing vesicles at the nerve terminal (Simpson, 2004; Schiavo et al., 1992;

Abbreviations: Botulinum type A toxin, BoNT/A; type C₁ toxin, BoNT/C₁; type E toxin, BoNT/E.

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Schiavo et al., 2000). Subsequently, the extra-cellular release of neurotransmitter into the neuromuscular junction is blocked and results in a flaccid muscular paralysis. The botulinum toxin light chains are zinc dependant endopeptidases that specifically cleave one of three soluble SNARE proteins. Type A, E and C₁ toxins cleave SNAP25_{1–206} (synaptosomal associated protein of molecular mass 25kDa), at positions Q¹⁹⁷–R¹⁹⁸, R¹⁸⁰–I¹⁸¹, and R¹⁹⁸–A¹⁹⁹ respectively. The remaining botulinum toxin serotypes cleave VAMP (vesicle associated membrane protein, or synaptobrevin) at positions Q⁷⁶–F⁷⁷, K⁵⁹–L⁶⁰, Q⁵⁸–K⁵⁹, and A⁸¹–A⁸² (Type B, D, F and G toxins respectively). Type C₁ toxin is the only serotype with two specific substrates which also cleaves Syntaxin at position K²⁵³–A²⁵⁴.

A dose of > 50ng of botulinum toxin per person typically causes a life-threatening, paralysing disease called botulism, however, low doses of the toxin are commonly used therapeutically to locally paralyse specific muscles for clinical or cosmetic benefit. Types A, B and E toxin are most commonly associated with naturally occurring botulism in humans. Type F is implicated less often, and types C and D are rarely associated with human botulism (Sharma et al., 2006; Demarchi et al., 1958; Prevot et al., 1955). There are, therefore, numerous reasons to test for the presence or the relative concentration of botulinum toxins.

Various different endopeptidase assays have been developed to specifically detect toxin enzymatic activity, however, many of these suffer from high variability, low sensitivity or the unwanted effects exerted by product specific excipients such as albumin (Hallis et al., 1996; Ekong et al., 1997; Anne et al., 2001). As the endopeptidase assays are uniquely specific for a specific toxin serotype, toxin identity is also obtained.

The principle of using a detecting antibody to a quadra, penta, hepta or octapeptide substrate sequence buried within the native substrates alpha-helix and unavailable for antibody binding before specific enzyme cleavage has only previously been described for botulinum type A, B and E toxins and expanded to tetanus toxin which also cleaves at Q⁷⁶–F⁷⁷ using the same detection system as for botulinum type B toxin (Hallis et al., 1996; Ekong et al., 1997; Sesardic et al., 2000; Shone et al., 2006). This concept was further developed for type A toxin to give a highly specific, sensitive, robust and reproducible assay. An octapeptide substrate sequence exposed following cleavage by type E toxin was utilised for the first time to obtain specific antibodies, providing additional evidence that this principle can be expanded to other specific enzyme cleavage reactions in the future.

2. Materials and methods

2.1. Synthetic SNAP25 peptide substrate

A 70 a.a. Synthetic SNAP25_{137–206} peptide (NH₂-VTNDA-RENEM-DENLE-QVSGI-IGNLR-HMALD-MGNEI-DTQNR-QIDRI-MEKAD-SNKTR-IDEAN-QRATK-MLGSG-OH) of > 80% purity with an expected mass of 7903.7Da and found mass of 7902.6 Daltons (by MALDI-TOF Mass Spec) was custom synthesized by Immune Systems Ltd, (Paignton, UK). A stock 4mg/ml solution of peptide in distilled water was aliquoted and stored frozen until required.

2.2. Full-length recombinant SNAP25

Full-length recombinant SNAP25_{1–206}, expressed as a fusion protein to glutathione-S-thioreductase and purified by affinity chromatography on glutathione-agarose after cleavage with thrombin was a generous gift from Dr J. Blasi (University of Barcelona, Spain).

2.3. Synthetic SNAP25 peptide immunogens

Synthetic SNAP25_{190–197}, SNAP25_{173–180}, and SNAP25_{193–206} with a Cysteine tag (C-TRIDEANQ), (C-TQNRQIDR) and (C-DEANQRATKMLGSG) respectively were custom synthesized by either Immune Systems Ltd, (Paignton, UK), or Sigma-Genosys (Cambridge, UK).

2.4. Primary detecting antibodies

BoNT/A cleavage site-specific anti-peptide SNAP25_{190–197}, antibody was raised in New Zealand White rabbits following conjugation to KLH through the Cys residue, as described previously (Ekong et al., 1997). Antibody purified by ammonium sulphate precipitation and dialysis was used at a concentration of ~ 11µg/ml in 2.5% w/v skimmed milk powder PBST throughout in microtitre plate assays. BoNT/E cleavage site-specific anti-peptide SNAP25_{173–180}, antibody was also raised in New Zealand White rabbits as described above and non-affinity purified antibody used at a concentration of 40µg/ml throughout in microtitre plate assays. Antibodies to SNAP25_{193–206} conjugated to KLH were raised in rabbits as described by Ekong et al. (1997).

2.5. Botulinum toxins

All concentrated botulinum toxins were safely handled within a Class I safety cabinet.

2.5.1. Botulinum type A toxins

Purified haemagglutinin free toxin was purchased from Metabio (USA) at 2.3×10^8 LD₅₀/mg, (1mg/ml from Hall strain) and diluted to 20,000 mouse LD₅₀/ml in Gelatine (0.2% w/v) Phosphate (50mM di-sodium hydrogen orthophosphate) Buffer pH 6.5 (GPB), aliquoted and stored frozen at -40°C . Haemagglutinin complex preparations were also used and vials of lyophilised material reconstituted immediately before use. Vials were carefully opened after removing the metal caps and piercing a hole through the rubber stoppers, using a fine needle ($0.5 \times 16\text{mm}$), to gently release the vacuum and stoppers carefully removed ensuring that none of the material was lost. Due to differences in the albumin concentrations in each of the reference or therapeutic preparations (500µg and $\sim 120\mu\text{g}$ per vial) different reconstitution buffers were used containing different amounts of BSA to give a final albumin concentration of 1mg/ml in the reconstituted material.

Candidate reference preparations both with (A19, from Hall strain 174) and without (A23, from Hall strain 174) the haemagglutinin complex, described previously in an International collaborative study with a geometric mean from 12 mouse LD₅₀ assays performed using GPB diluent of 1831 and 650 mouse LD₅₀/vial respectively (Sesardic et al., 2003).

2.5.2. Botulinum type E toxin

Purified haemagglutinin free toxin was purchased from Metabio (USA) at 1mg/ml (3×10^5 LD₅₀/mg pre-trypsinised, from Alaska strain) and trypsinised. One part (by protein weight) of trypsin (Type XIII-TPCK treated, Sigma T8642) was added to 20 parts toxin in 0.1M sodium phosphate buffer pH 7.0, incubated for 30min at 37°C , and the reaction stopped by adding a 2× concentration of soya bean trypsin inhibitor (Sigma, T-9003). Activated toxin was then diluted 1 in 2000 in GPB, aliquoted and stored frozen at -80°C . On thawing this material contained 13,617 mouse (outbred MF1) LD₅₀/ml (40.9×10^6 LD₅₀/mg trypsinised toxin).

2.5.3. Botulinum type C₁ toxin

Purified haemagglutinin complex toxin was purchased from Metabio (USA) at 1mg/ml (4×10^6 LD₅₀/ml, from Brazil strain) and diluted to 10k mouse LD₅₀/ml in GPB, aliquoted and stored frozen at -40°C .

2.6. SDS-PAGE and Western blotting

The proteolytic activity of botulinum type A and E neurotoxins on recombinant SNAP25_{1–206} was examined by Western blotting. Purified, botulinum type A

(133nM) or trypsinised botulinum type E (133nM) was reduced with 10mM DTT (30min at 37°C) and reacted with 50µg SNAP25 (50mM HEPES, 20µM ZnCl₂, pH 7.0, 2mM DTT) for 2h at 37°C . Cleavage mixtures were separated by SDS-PAGE, using 4–20% gradient gels (Bio-Rad) and visualised by Western blotting (Ekong et al., 1997). Total protein blots were blocked with 0.3% v/v Tween 20 (polyoxyethylene sorbitan monolaurate) in PBS (Phosphate buffered saline: 137mM NaCl, 1.5mM KH₂PO₄, 8.0mM Na₂HPO₄·2H₂O, 2.7mM KCl) and stained with colloidal gold. Immunoblots were incubated with 5µg/ml of the affinity purified targeted antibodies. Goat anti-species IgG, coupled to HRP (Sigma) was used as the second antibody at a dilution of 1/2000. Antibody dilutions were made in 2.5% skimmed milk in PBST (PBS containing 0.05% v/v Tween 20), and incubated for 90min at 37°C . Protein bands were visualised with Sigma Fast DAB (diaminobenzidine) substrate system. Tris–tricine SDS-PAGE (16.5%) was performed as described by Schagger and von Jagow (1987) to analyse SNAP25_{137–206} samples. Botulinum type E toxin (50,000 LD₅₀/ml) was reacted with 1mg/ml SNAP25_{137–206} in 50mM HEPES, 20µM ZnCl₂, pH 7.0, either with or without the presence of 4mM DTT for 3h at 37°C . Samples were diluted 1 in 4 in sample buffer, heated at 65°C for 2min and run at 100v. Gels were fixed with 5% glutaraldehyde and stained with 0.025% Brilliant blue G in 10% acetic acid.

2.7. Immobilisation of synthetic SNAP25_{137–206} peptide

Polystyrene 96 well plates (Nunc Maxisorp) were coated with 100µl/well of either 2 or 3µg/ml SNAP25_{137–206} substrate in 50mM carbonate buffer (pH 9.6) overnight at 4°C or room temperature (RT, in a sealed box), washed (×3) with PBST or decanted and blocked with 300µl/well of 5% skimmed milk powder PBST for 90min at 37°C or RT. Plates were then washed 3 times with distilled water and air dried and either used immediately or stored sealed with a dry desiccator capsule at -20°C .

2.8. Toxin endopeptidase assay

Toxins were suitably diluted in reaction buffer and doubling dilutions performed on a SNAP25_{137–206} substrate coated plate in reaction buffer (50mM HEPES, 20µM ZnCl₂, pH 7.0) containing 1mg/ml BSA (fatty acid free, globulin free, Sigma, A0281), 5 or 2.5mM DTT for type A and E toxins respectively and the indicated Tween 20 concentration. Plates were then individually sealed and incubated at either 37°C or RT

as indicated without stacking the plates for 1.5, 18, 24 or 48h as indicated. Plates were then washed 3 times with PBST, blot dried, and 100µl/well of primary detecting antibody (either: anti-SNAP25_(190–197) or anti-SNAP25_(173–180) for toxin A and E respectively) in 2.5% (w/v) skimmed milk powder PBST (antibody buffer) added, sealed individually and incubated at either 37°C or RT for 90min without stacking.

Plates were washed again and 100µl/well of goat anti-rabbit–HRP conjugate (Sigma A0545, 1 in 2000 dilution in antibody buffer) added and incubated as before. After washing 100µl/well substrate solution (50mM citric acid pH 4.0, 0.05% w/v ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, and 0.05% v/v of a 30% w/v Hydrogen peroxide solution) was added and the colour allowed to develop at RT for 30–60min. Following colour development the plates were briefly shaken and absorbance read at 405nm using a suitable plate reader (Multiscan plate reader).

2.9. Calculation of relative activity

Relative activities in test samples were calculated with respect to a reference sample included on the same plate, by parallel line analysis. Three or four sequential dilutions from the linear dose range of reference and test sample were used for parallel line analysis. Parallel line analysis was only valid if all the following criteria were fulfilled: There was a significant regression on dose response ($p < 0.01$), and no significant deviations from parallelism or linearity at the 1% level. All relative activities are shown with 95% confidence limits, unless otherwise indicated.

3. Results

3.1. Western blot analysis

Total protein blots showed that both toxins cleaved SNAP-25_{1–206} with the production of lower molecular weight products (Fig. 1a). Only non toxin treated SNAP25_{1–206} was recognised by an antibody (anti-SNAP25_{193–206}) selective for the intact substrate (Fig. 1b), confirming that all of the SNAP25 had been cleaved by the respective toxins. A band representing SNAP25_{1–197} was evident following BoNT/A cleavage and was only detected with anti-SNAP25_{190–197}. Cleavage by BoNT/E produced two major products of which only the higher MW predominant component was selectively recognised by anti-SNAP25_{173–180} representing SNAP25_{1–180} and a lower MW unknown component between 14 and 22kDa.

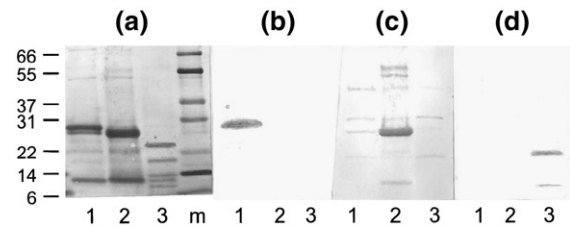


Fig. 1. Western blotting characterisation of SNAP25 toxin cleavage. Colloidal gold staining (a), and Western blotting using antibodies targeted to intact SNAP25_{193–206} (b), BoNT/A cleavage site SNAP25_{190–197} (c), and BoNT/E cleavage site SNAP25_{173–180} (d). Lane 1: Intact SNAP25_{1–206}; lane 2: BoNT/A treated SNAP25_{1–206}; lane 3: BoNT/E treated SNAP25_{1–206}. (MW markers shown in kDa).

3.2. Preliminary type A endopeptidase optimisation

It was found that the pre-activation/reduction step described previously (Ekong et al., 1997; Schmidt et al., 2001) could be conveniently eliminated provided the time conventionally used for the pre-activation step was added to the reaction time on the SNAP25_{137–206} coated plate (e.g. 90min vs 30min + 60min) in the presence of an optimal 5mM DTT (data not shown) and was therefore used throughout in these studies. No type A activity was found when DTT was omitted from all steps.

3.3. Preliminary type E endopeptidase optimisation

Reagents were optimised for the type E assay and the following conditions adopted: 2.5mM DTT reaction buffer in the absence of NaCl (Fig. 2a, b) and cleavage specific antibody anti-SNAP25_(173–180) at 40µg/ml. No pre-activation step was required for type E toxin and it was unexpectedly discovered that in the absence of any DTT reduction step that 60% optimal endopeptidase activity was still evident in the absence of Tween 20 (Fig. 2a). Like type A, type E toxin was also found to have an optimal pH of 7.0 (data not shown).

3.4. Tris–tricine SDS-PAGE analysis

SNAP25_{137–206} substrate was predominantly cleaved after 3h at 37°C by BoNT/E both in the presence and absence of DTT and produced two identical cleavage products (Fig. 3).

3.5. Effects of Tween 20, albumin and coating concentration

In the absence of albumin a large increase in endopeptidase activity was seen, even with low concentrations of

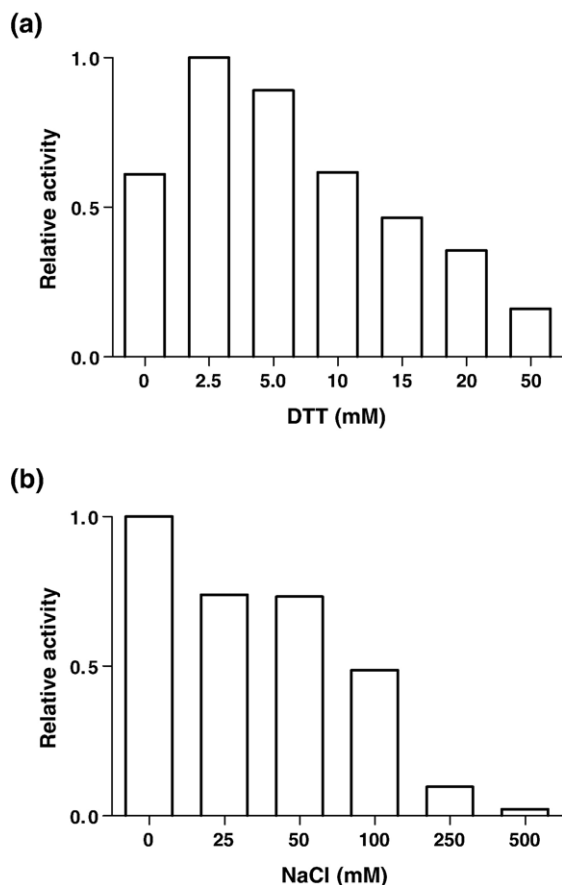


Fig. 2. Preliminary optimisation of type E endopeptidase activity. Effect of DTT (a), and NaCl (b) concentrations on specific microtitre plate endopeptidase activity.

just 0.05% Tween 20 but which then plateaued off with higher concentrations (Fig. 4a).

An increased BoNT/A enzymatic activity was also found with albumin (1mg/ml), but further increases were also evident when a combination of albumin with various Tween 20 concentrations were used (Fig. 4b, c).

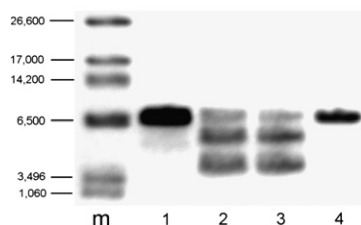


Fig. 3. Tris-Tricine SDS-PAGE (16.5%) gel of the effects of DTT on BoNT/E toxin substrate cleavage. Lane 1: SNAP25_{137–206} (2.5 µg) treated in the presence of DTT; lane 2: SNAP25_{137–206} (2.5 µg) treated in the presence of DTT with BoNT/E; lane 3: SNAP25_{137–206} (2.5 µg) treated in the absence of DTT with BoNT/E; lane 4: SNAP25_{137–206} (1.25 µg) treated in the presence of DTT. (MW markers shown in Da).

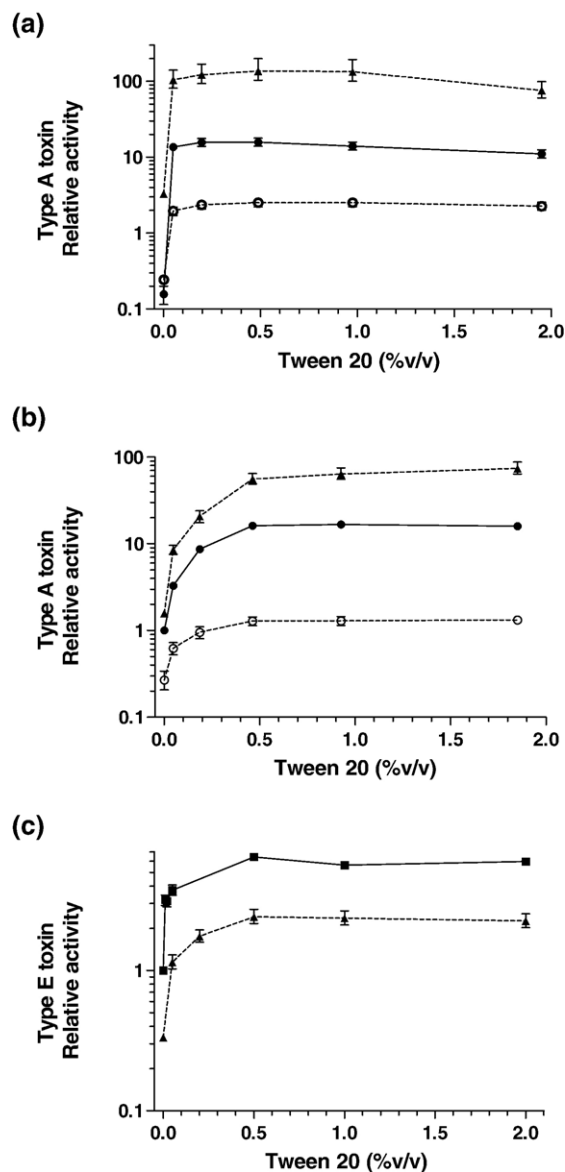


Fig. 4. Effects of Tween 20 concentrations on microtitre plate endopeptidase activity. In the absence (a) and presence (b, c) of 0.1% BSA. Reaction conditions were 90 min at either 37 °C (a, b, solid line, solid circle) or RT (a, b, broken line, open circle), and 18 h at either RT (a, b and c, broken line, triangles) or 37 °C (c, square symbols solid line).

Unlike in the absence of albumin there was a gradual increase with increasing Tween 20 concentrations until it plateaued off at 0.5%. Despite double the sensitivity being achieved with Tween 20 alone, it was found to be more variable with a greater margin of error than the combination of albumin and Tween 20 (Fig. 4a, b).

An optimal effect was produced with 0.5% v/v Tween 20 in the reaction buffer and an optimal substrate coating concentration of 2–4 µg/ml (Fig. 5). A greater

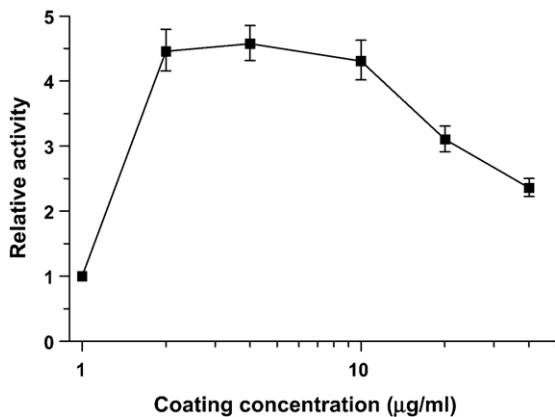


Fig. 5. Effect of substrate (SNAP25_{137–206} peptide) coating concentration. Botulinum type A toxin assayed for 90 min at 37 °C with 0.5% Tween / 0.1% BSA on SNAP25 substrate coated plates.

than 4 fold reduction was however, evident when the coating concentration was reduced from 2 to 1 µg/ml, therefore, for the robustness of the assay 3 µg/ml of substrate was used for coating in further experiments. A similar difference was found with BoNT/E at different Tween 20 concentrations, again with optimal effects produced with 0.5% v/v (Fig. 4b). Under these optimal conditions but in the absence of DTT 60% BoNT/E endopeptidase activity was still obtained (data not shown).

3.6. Effects of time, temperature and specificity

Although a 90min incubation at 37°C was adequate for most uses a 1.4 fold higher sensitivity was obtained by incubating for 18h at 37°C for type A toxin compared

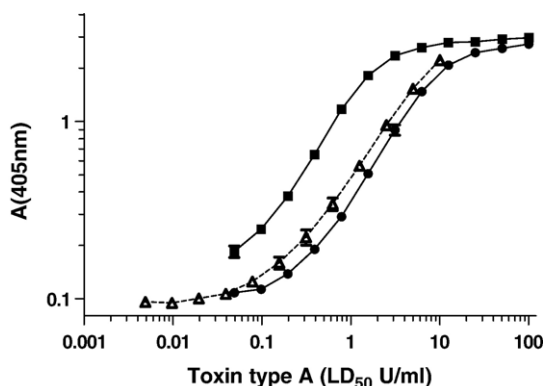


Fig. 6. Effects of temperature and time on type A endopeptidase activity in the presence of 0.5% Tween 20 and 0.1% albumin. Reaction for 18 h at either RT (squares solid line), or 37 °C (triangles broken line), or 90 min at 37 °C (circle, solid line). $\pm$ SD ($n=4-6$).

to a 5.6 fold higher sensitivity when incubated for 18h at RT (Fig. 6). No significant further increase or decrease in sensitivity was seen after 24 or 48h at RT for type A toxin with relative activities of 1, 1.03 (0.96–1.10), 1.11 (1.04–1.19) at 18, 24 and 48h respectively with 95% limits shown in brackets. No significant relative difference by parallel line analysis was found between A19 and A23 (1.09, 95% limits: 0.98–1.22) toxins using the collaborative study average LD₅₀ values determined using GPB as diluent (Sesardic et al., 2003), however, the Metabiotics toxin gave a slightly lower relative value of 0.88 (0.79–0.99) using the manufacturers provided LD₅₀ value before in house dilution and frozen storage (Fig. 7). Despite just one a.a. difference in the cleavage sites between A and C₁ toxins, only minimal cross reactive binding was found between toxin C₁ cleaved SNAP25 and anti-SNAP25_{190–197} antibody (Fig. 7). When doubling dilutions of type A, E and C₁ toxins were allowed to cleave the SNAP25_{137–206} substrate for 18h at RT, washed (distilled water) and an intermediate type A toxin concentration (0.5 LD₅₀/ml) applied to all the wells for 18h at RT and then probed with the anti-SNAP25_{190–197} antibody a partial inhibitory effect was produced by high concentrations (100 LD₅₀/ml) of type C₁ and E toxins, and a potentiating effect by the control type A toxin concentrations (data not shown).

Incubating at 37°C for longer than 90min resulted in a 1.29 (2h), 1.32 (3h), and 3.41 (18h) fold increase in type E activity. Unlike with type A the sensitivity for BoNT/E was an order of magnitude less and unlike with BoNT/A near maximal sensitivity (3 fold higher) was obtained by incubating for 18h at 37°C compared to 18h

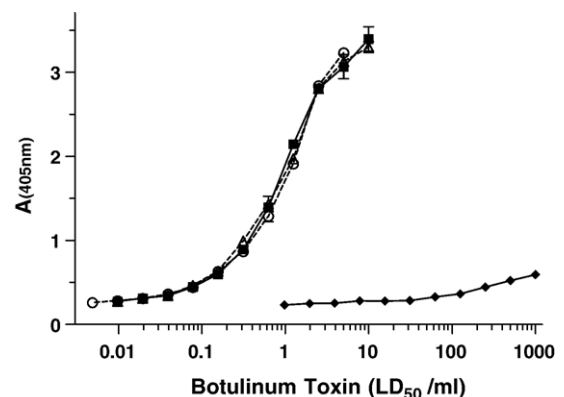


Fig. 7. Specificity of anti-SNAP25_{190–197} detection antibody. Endopeptidase activity of toxin A with and without haemagglutinin complex and type C toxin. Toxin A haemagglutinin complex (A19, solid line/squares), haemagglutinin free toxin A (broken lines: A23 toxin, triangle, and Metabiotics toxin, circle). Toxin C (diamonds).

at RT (Fig. 4b). Unlike with type A, BoNT/E sensitivity was further increased to 152% (131–179) after 24h at 37°C but decreased to 45% (39–52) at 48h relative to 100% at 18h.

Less variability was found within the plate when all incubation steps were performed at RT and was, therefore, adopted throughout, where possible.

It was also found that anti-SNAP25_{193–206} (at a 1 in 100,000 dilution) bound both SNAP25_{137–206} substrate coated wells treated both with and without toxin A or C (data not shown).

3.7. Limit of detection

The background mean + 2SD gave a limit of detection of 0.01 LD₅₀/ml with all the toxin A haemagglutinin free preparations (A23 and Metabiolecs toxin) and haemagglutinin complexed toxins (e.g. A19, see Fig. 8a) tested when incubated at RT for 18h. Toxin E gave a limit of detection of less than 0.2 LD₅₀/ml when incubated at 37°C for 18h (Fig. 8b).

3.8. Assay precision

Plates were coated with 3 µg/ml substrate overnight at RT decanted and blocked at RT. Assays were performed with incubation temperatures and times indicated in Section 3.7, and using replicated duplicate toxin dilution curves the relative activities were calculated against columns 7 and 8 on each microtitre plate, with 4 plates per assay, with further incubation steps all performed at RT. Assay precision in terms of the geometric coefficient of variation (GCV) were calculated as ratios of the transformed log standard deviations to the geometric means, with typical optimised type A assays (all incubations performed at RT) giving an intra-plate GCV ranging from 1 to 6%, and an inter-plate GCV of 2 to 8%. Inter-assay variation was < 2% (GCV) between five separate assays performed on different weeks. A similar inter-plate GCV of 3.8% was also obtained with the type E assay.

4. Discussion

Highly specific SNAP25 cleavage site (190–197; 173–180) detecting antibodies were utilised in these studies to develop sensitive, highly reproducible endopeptidase immuno-assays. The specific nature of the detecting antibody was clearly demonstrated by the failure of anti-SNAP25_{190–197} to recognise the BoNT/C₁ cleavage product which differs by just one aa residue. The high relative molecular weights of SNAP25 and its

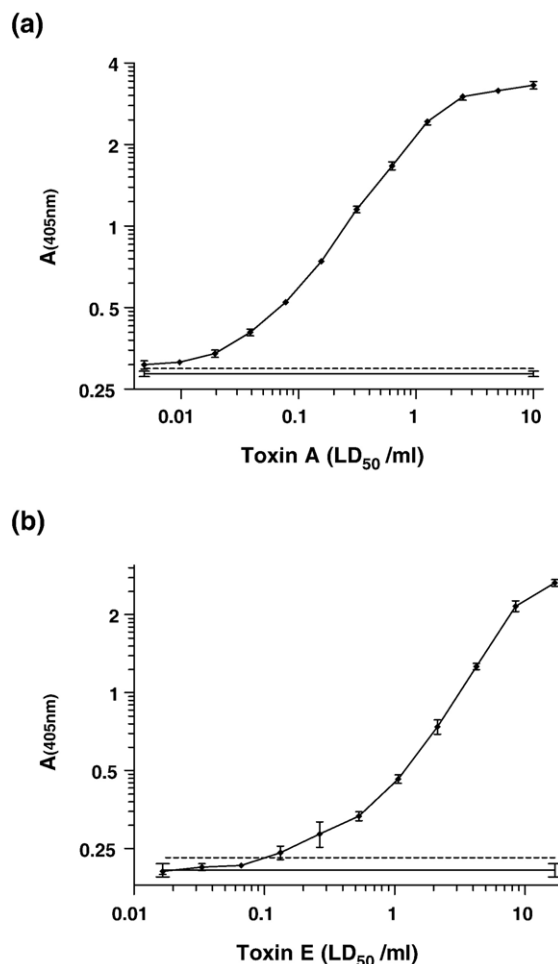


Fig. 8. Endopeptidase assay limits of detection. Type A haemagglutinin complex toxin (A19) incubated for 18 h at RT (a), or type E toxin incubated for 18 h at 37 °C (b). Toxin A or E toxins ($\pm$ SD, $n=6$). Negative control ($n=24$) and limit of detection (mean negative control + 2SD) broken line.

identified cleaved fragments (1–197 and 1–180) seen in Fig. 1 have previously been shown using SDS-PAGE gels (Schiavo et al., 1993; Binz et al., 1994; Bajohrs et al., 2004). However unlike these authors a second lower MW component could also be seen following BoNT/E cleavage which was not detected by the SNAP25_{173–180} or SNAP25_{193–206} antibodies. Two cleavage bands were also reported by Lawrence et al. (2007) using SNAP25_{142–206} substrate and produced by a purified recombinant type E toxin preparation. Using the microtitre plate assay it was also possible to demonstrate the type E toxin cleavage product SNAP25_{137–180} was further cleaved as indicated by the reduced assay sensitivity or decreased anti-SNAP25_{173–180} binding following prolonged incubations of 48h perhaps indicating a second

cleavage point at the C-terminal end of SNAP25_{137–180}. Previously described contaminating bacterial enzymes may be unaffected by normal trypsin inhibitors (soya bean trypsin inhibitor and PMSF) as used by Lawrence et al. (2007) and ourselves (Dekleva and Dasgupta, 1990; Ohishi et al., 1975). However, some of these are known to be dependent on DTT reducing conditions, which is in contrast to the current findings where production of the two bands was found to be independent of DTT (Dekleva and Dasgupta 1990, Ohishi et al., 1975). Although the current studies failed to identify any contaminating enzymes its presence cannot be excluded.

Despite anti-SNAP25_{193–206} only detecting un-cleaved SNAP25_{1–206} on Western blots, the same antibody could not distinguish between microtitre wells coated with SNAP25_{137–206} and either treated with or without toxin, indicating that both the N and C terminals of the substrate are bound to the plates. However, Sharma and Singh (2004) using the complete SNAP25_{1–206} sequence coated at 10 µg/ml at pH 7.2 were able to monitor type A and E cleavage with a similar C-terminal specific (anti-SNAP25_{195–206}) antibody, indicating that under these conditions the C-terminal was not bound to the plates.

Although type A endopeptidase assays utilising antibodies to either 5, 7 or 8 a.a. of the exposed cleavage site of SNAP25 have previously been described (Hallis et al., 1996; Ekong et al., 1997; Shone et al., 2006), these assays lacked the desired sensitivity (0.13 ng/ml and 0.1–0.8 LD₅₀/ml respectively) or reproducibility ideal for testing of product lots or other routine purposes (Hallis et al., 1996; Gaines Das et al., 1999). For instance, therapeutic toxin products are subject to regulatory requirements stipulating that each batch of product must contain between 80–125% of the label value with 95% confidence limits (CL) no greater than 80–125% of the calculated potency (PhEur monograph, 2005). The following studies were therefore, instigated to increase the sensitivity and reproducibility of the assay and develop a new endopeptidase assay for type E toxin. Numerous changes were made to the previously described assay for type A (Hallis et al., 1996; Gaines Das et al., 1999; Ekong et al., 1997; Schmidt et al., 2001), to remove any steps likely to introduce errors into the assay and other SNAP25 specific toxins investigated. The pre-activation/reduction step previously described could be conveniently eliminated, thereby simplifying and eliminating one potential source of variability. Assay sensitivity was increased 50 fold following these changes, together with the use of an optimal 0.5% Tween 20 concentration in tandem to 0.1% albumin. Assay variability as described by Gaines Das et al. (1999) was also greatly reduced following the various improvements from the previously reported intra-

plate GCV of 6–12% to 1–6% and for between plates from 11% to 2–8%. The between assay GCV was also reduced from the previously reported 25% to <2% indicating that this newly described assay has a significantly improved level of precision more suitable for batch release monitoring of type A toxin in therapeutic products.

Under the assay conditions it was also found that BoNT/E was more stable at 37 °C than BoNT/A for which optimal activity was found at RT and was therefore adopted to reduce assay variability. This appears to be in agreement with the high temperature stability of type E light chain and low thermal stability of type A light chain previously reported (Blanes-Mira et al., 2001; Ibanez et al., 2004). As Blanes-Mira et al. (2001) have shown that recombinant type E light chain was still active after incubating for 1 h at up to 55 °C, the sensitivity of this assay may, therefore, be further increased or reaction times reduced by using higher incubation temperatures.

The newly developed type A assay has a limit of detection equivalent to that described by Chao et al. (2004) for an immuno-polymerase chain reaction assay and Kalb et al. (2006) for an endopeptidase mass-spec assay, but better than that described for capture ELISA, mouse bioassays or SNAPtide or other fluorescent type assays (Ekong et al., 1995; Kautter and Solomon, 1977; Schmidt et al., 2001; Schmidt and Stafford, 2003a,b; Sesardic et al., 2004; Jones et al., 2006).

Higher titre/high avidity anti-SNAP25_{190–197}/SNAP25_{173–180} cleavage site detecting antibodies would, however, considerably increase the sensitivity of the current assays and if produced in sheep, large quantities would become available at a minimal cost (Avrameas, 1992; Landon and Smith, 2003). As both the N and C substrate (SNAP25_{137–206}) terminals are bound to the ELISA plates, it may be possible to further increase sensitivity by using a combination of antibodies raised to octapeptides from both sides of the cleavage site, thereby potentially doubling the signal. The use of affinity purified antibodies or an amplification system could also be used for further enhanced sensitivity if required (Hallis et al., 1996; Avrameas, 1992).

This assay system offers the convenience of conventional immuno-assays without the need for expensive specialist equipment, making it easily transferable. When used with a product specific reference manufactured from the same bulk of active substance, activity in terms of relative LD₅₀ units can be monitored in the final product lots, often with tighter 95% CL's than those obtained using large numbers of mice, thereby potentially saving animals.

In summary this work describes an easily transferable, highly specific, sensitive, robust and reproducible

endopeptidase immunoassay suitable for monitoring BoNT/A products and its preliminary development for use with BoNT/E.

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