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Recombinant SNAP-25 is an effective substrate for *Clostridium botulinum* type A toxin endopeptidase activity *in vitro*

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Bacterial neurotoxins are now being used routinely for the treatment of neuromuscular conditions. Alternative assays to replace or to complement *in vivo* bioassay methods for assessment of the safety and potency of these botulinum neurotoxin-based therapeutic products are urgently needed. Advances made in understanding the mode of action of clostridial neurotoxins have provided the basis for the development of alternative mechanism-based assay methods. Thus, the identification of SNAP-25 (synaptosomal-associated protein of molecular mass 25 kDa) as the intracellular protein target which is selectively cleaved during poisoning by botulinum neurotoxin type A (BoNT/A) has enabled the development of a functional *in vitro* assay for this toxin. Using recombinant DNA methods, a segment of SNAP-25 (aa residues 134–206) spanning the toxin cleavage site was prepared as a fusion protein to the maltose-binding protein in *Escherichia coli*. The fusion protein was purified by affinity chromatography and the fragment isolated after cleavage with Factor Xa. Targeted antibodies specific for the N and C termini of SNAP-25, as well as the toxin cleavage site, were prepared and used in an immunoassay to demonstrate BoNT/A endopeptidase activity towards recombinant SNAP-25 substrates. The reaction required low concentrations of reducing agents which were inhibitory at higher concentrations as were metal chelators and some inhibitors of metallopeptidases. The endopeptidase assay has proved to be more sensitive than the mouse bioassay for detection of toxin in therapeutic preparations. A good correlation with results obtained in the *in vivo* bioassay ($r = 0{\cdot}95$, $n = 23$) was demonstrated. The endopeptidase assay described here may provide a suitable replacement assay for the estimation of the potency of type A toxin in therapeutic preparations.

Keywords: botulinum neurotoxin type A, endopeptidase assay, recombinant SNAP-25, targeted antibodies

INTRODUCTION

Botulinum and tetanus neurotoxins are potent neuroparalytic agents which inhibit cholinergic neurotransmission, thereby causing the flaccid and spastic paralyses of botulism and tetanus, respectively (Simpson, 1981; Sakaguchi, 1983). The specific action of botulinum neurotoxin type A (BoNT/A) is now being exploited in the treatment of certain neuromuscular conditions (Jankovic & Brin, 1991). The therapeutic preparation consists of minute (ng) quantities of the toxin protein non-covalently associated to form a high molecular mass complex (900 kDa) with non-toxin protein and haemagglutinin components, together with mg quantities of human serum albumin added as bulking agent. The increasing use of BoNT/A presents a number of technical challenges for the measurement of toxin potency in therapeutic preparations. Potency assays for the toxin are critically important for the standardization and control of these substances and, at present, the

Abbreviations: BoNT/A, botulinum neurotoxin type A; HRP, horseradish peroxidase; MBP, maltose-binding protein; SNAP-25, synaptosomal-associated protein of molecular mass 25 kDa; VAMP, vesicle-associated membrane protein.

mouse bioassay is the only method sufficiently sensitive to determine the activity of BoNT/A in therapeutic preparations and is the only assay accepted by the regulatory authorities for this purpose. As such, the assay has been identified as a priority target for replacement by the European Centre for the Validation of Alternative Methods (ECVAM) and FRAME (Fund for the Replacement of Animals in Medical Experiments) (Balls & Straughan, 1996). A number of alternative bioassays are under development which rely on less severe end-points, such as the non-systemic local effect of botulinum toxin described by Pearce *et al.* (1995) and Sesardic *et al.* (1996). However, these assays still depend on the use of animals. It is highly desirable to replace these with assays which avoid the use of animals without compromising sensitivity.

Over the last few years, a number of *in vitro* bioassays for clostridial neurotoxins have been developed. Many of these are immunoassays with sensitivities approaching that of the conventional bioassay, employing either highly specific and sensitive monoclonal antibodies (Shone *et al.*, 1985), or sophisticated amplification systems such as evanescent-wave immunosensors (Kumar *et al.*, 1994) and solid-phase coagulation (Doellgast *et al.*, 1993) or which rely on detection of toxin in the complexed form (Singh *et al.*, 1996). We have also developed a highly sensitive and simple ELISA for the detection of minute quantities of BoNT/A in therapeutic preparations (Ekong *et al.*, 1995). Our studies confirmed that non-functional immunoassays are limited by their inherent inability to differentiate between biologically active and inactive toxin.

Clostridial neurotoxins are now known to be zinc-dependent metalloendopeptidases (Montecucco & Schiavo, 1993, 1995) which block the release of neurotransmitters by the specific cleavage of one of the proteins forming the core of the synaptic vesicle docking-fusion complex (Söllner *et al.*, 1993). Thus, tetanus and type B toxins cleave the second isoform of vesicle-associated membrane protein (VAMP, also known as synaptobrevin) at the peptide bond Gln_{76}–Phe_{77} (Schiavo *et al.*, 1992; Link *et al.*, 1992). BoNT/A cleaves synaptosomal-associated protein of molecular mass 25 kDa (SNAP-25) at Gln_{197}–Arg_{198} (Blasi *et al.*, 1993; Schiavo *et al.*, 1993a, b; Binz *et al.*, 1994). This action of BoNT/A provides a basis for the development of a functional *in vitro* assay for the toxin utilizing its endopeptidase activity. Such an assay requires a convenient source of purified target protein for use as substrate and a means of monitoring the cleavage of substrate. A number of recent studies have demonstrated that synthetic peptide fragments of VAMP (>30 aa) may be used as *in vitro* substrates to examine the endopeptidase activity of tetanus and type B neurotoxin (Shone & Roberts, 1994; Foran *et al.*, 1994). These studies suggest that clostridial neurotoxins require large peptide substrates for the expression of their enzymic activity and that amino acid residues distal from the cleavage site may have an influence on the reaction (Wictome *et al.*, 1996; Soleilhac *et al.*, 1996).

This study describes the preparation of a recombinant fragment of SNAP-25 ($SNAP\text{-}25_{134–206}$) spanning the toxin cleavage site and its use as an *in vitro* substrate for BoNT/A. The recombinant substrate and targeted antibodies were used in an immunoassay to characterize the endopeptidase activity of BoNT/A in purified and therapeutic preparations. The activity of therapeutic preparations of BoNT/A was compared using the endopeptidase assay and the mouse bioassay.

METHODS

Reagents. A mouse cDNA clone, pSNAP8.52, containing the entire coding region of SNAP-25 was kindly provided by Dr M. C. Wilson of the Scripps Research Institute, La Jolla, CA, USA (Oyler *et al.*, 1989). The expression vector pMAL-c2, amylose resin, restriction endonucleases and Factor Xa were purchased from New England Biolabs and were used as recommended by the manufacturer. Highly purified BoNT/A (specific activity $1{\cdot}56 \times 10^8$ mouse LD_{50} mg^{-1}) was the kind gift of Dr C. C. Shone, CAMR, Porton Down, Salisbury, UK. Other SNAP-25-specific peptides ($SNAP\text{-}25_{1–14}$, $SNAP\text{-}25_{190–197}$ and $SNAP\text{-}25_{193–206}$) were custom prepared by Genosys Biotechnologies. Except where specified, other reagents were purchased from Sigma. The *Escherichia coli* strain TB1 [*ara* Δ(*lac–proAB*) *rpsL* (ϕ80 *lacZ*ΔM15) *hsdR*] was used as the host for expression of plasmids.

Construction and expression of SNAP-25 fragment as a fusion protein. The nucleotide sequence encoding a region of SNAP-25 spanning the toxin cleavage site (corresponding to aa residues 134–206) was amplified by PCR using the forward primer 5′ GCCATCAGTGGTGAATTCATCCGCAGGGTA and the reverse primer 5′ ACTGATCAAGCTTAACCACT-TCCCAGCATC and cloned in the pMAL-c2 expression vector between the *Eco*RI and *Hin*dIII sites (underlined) such that the sequence encoding the SNAP-25 fragment (GenBank accession no. M22012) was in-frame with the *malE* gene of *E. coli* (Fig. 1). Expression was under the control of the P_{tac} promoter and the vector encoded the Factor Xa recognition

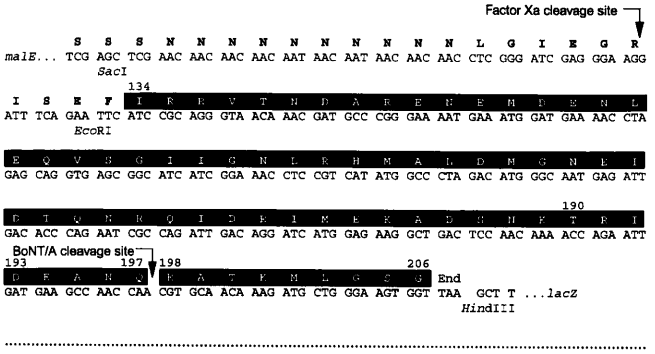

Fig. 1. Cloning and expression of SNAP-25 fused to the C terminus of MalE. The nucleotide sequence encoding the SNAP-25 amino acid sequence from residue 134 to 206 was cloned between the *Eco*RI and *Hin*dIII sites at the polylinker of the vector pMAL-c2. The vector-derived nucleotide sequence encoding the linker peptide added to the C terminus of MalE is shown, including the factor Xa cleavage site used in the purification of the SNAP-25 peptide from MalE. The position of the BoNT/A cleavage site on the SNAP-25 peptide is also indicated. The gene fusion protein was under the control of a P_{tac} promoter.

site between *malE* and the fused SNAP-25 gene fragment to facilitate subsequent purification of the peptide. The SNAP-25-maltose-binding protein (SNAP-25-MBP) fusion protein was expressed by inoculating 1 litre tryptone-yeast broth containing 2 mg glucose ml^{-1} and 100 μg ampicillin ml^{-1} with overnight cultures of *E. coli* cells containing the plasmid. Cells were grown at 37 °C and expression of fusion protein was induced by the addition of 1 mM IPTG when the OD_{600} of the culture was about 0·6. Bacteria were harvested by centrifugation (10 min at 3000 *g*) 2–24 h after induction. Longer induction times (up to 24 h) improved the yield and did not affect stability of the fusion protein. Pellets were resuspended in 20 mM Tris/HCl, pH 7·0, containing 0·2 M NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT and 1 mM sodium azide (Buffer A) and frozen at −20 °C until used. Frozen cells were lysed by sonication (Neu & Heppel, 1965) and the crude extract collected after centrifugation at 4 °C for 30 min at 10000 *g*.

Purification of the recombinant SNAP-25 fusion protein and SNAP-25 fragment. Cell sonicates containing SNAP-25-MBP fusion protein were resuspended in Buffer A and applied to an amylose affinity column (2·5 × 10 cm, 40 ml resin). Unbound material was washed off the column with Buffer A and bound material was specifically eluted with Buffer A containing 10 mM maltose (Buffer B). Column fractions were monitored by A_{280} and SDS-PAGE. Samples containing fusion protein were concentrated to 1 mg ml^{-1} (Amicon Centricon concentrator) and cleaved for 24 h at room temperature with Factor Xa in Buffer B using a ratio of 0·5 % (w/w) Factor Xa to the fusion protein. The recombinant SNAP-25 fragment was then purified by ultracentrifugation using a Centricon 30 kDa cut-off membrane filter, followed by a Centricon 3 kDa cut-off membrane filter.

Preparation of synaptosomes. Crude synaptosomes were prepared from rat brain by differential sucrose density centrifugation, as described by Huttner *et al.* (1983).

Production and characterization of antipeptide antibodies. Synthetic peptides of SNAP-25 (corresponding to aa residues 1–14, 190–197 and 193–206) were coupled to keyhole limpet haemocyanin and used to produce the site-specific antibodies R1, R2 and R3, respectively, in rabbits. Adult female New Zealand White rabbits (2–3 kg) were injected sub-cutaneously with four doses of each immunogen (300 μg per dose) in PBS. Primary injections were carried out in an equal volume of complete Freund's adjuvant and booster injections were carried out in PBS at 4-week intervals. Immune sera were harvested 10 d after the final injection and purified immunoglobulin prepared by precipitation in ammonium sulphate followed by Protein G affinity chromatography (Pharmacia). Affinity-purified IgG was also prepared using antibody-specific affinity columns prepared by coupling the specific peptide antigen to CNBr-activated Sepharose (Sigma). Antibodies were screened for specificity by Western blotting against crude synaptosomal extracts before and after treatment with purified BoNT/A, and by ELISA using peptide-coated microtitre plates and goat-anti rabbit IgG conjugated to horseradish peroxidase (HRP) (Sigma).

Cleavage of synaptosomal and recombinant SNAP-25 by BoNT/A. The ability of BoNT/A to cleave SNAP-25 in synaptosomes, recombinant $SNAP\text{-}25_{134\text{–}206}$-MBP fusion protein (SNAP-25-MBP), or the recombinant fragment (r-$SNAP\text{-}25_{134\text{–}206}$) was examined. Purified BoNT/A (0·5 nM or 0·013 nM), reduced by incubation for 30 min at 37 °C with 10 mM DTT or 20 mM β-mercaptoethanol in 100 mM HEPES, pH 7·4, containing 1·0 mM NaCl, 20 μM $ZnCl_2$ was used to treat 200 μg crude synaptosomes (solubilized with 1 % *n*-octyl-β-D-glucopyranoside, 200 μg SNAP-25-MBP or 50 μg r-$SNAP\text{-}25_{134\text{–}206}$). The final reaction volume of 400 μl was incubated at 37 °C and after specified periods of time, 50 μl aliquots were transferred to an equal volume of 0·2 M HCl stop solution. Samples were stored at −20 °C prior to analysis by SDS-PAGE and Western blotting.

Characterization of cleavage products. Cleavage mixtures were analysed by SDS-PAGE (Laemmli, 1970) and Western blotting (Towbin *et al.*, 1979), using 10–20 % (v/v) gradient gels. Total protein blots were blocked with PBS containing 0·3 % Tween 20 and stained with colloidal gold. Immunoblots were blocked with 5 % (w/v) skimmed milk (Marvel) in PBS containing 0·05 % Tween 20 (PBST) and incubated with 1 μg of either the R3 or R2 affinity-purified polyclonal antibodies ml^{-1}, specific either for the parent substrate ($SNAP\text{-}25_{1\text{–}206}$, SNAP-25-MBP or r-$SNAP\text{-}25_{134\text{–}206}$) or for the N-terminal cleavage product ($SNAP\text{-}25_{1\text{–}197}$ or $SNAP\text{-}25_{134\text{–}197}$), respectively. The second antibody (affinity-purified goat anti-rabbit IgG coupled to HRP) was used at a dilution of 1/2000 and immobilized antigens were visualized with the Sigma Fast DAB (diaminobenzidine) substrate system.

Cleavage products were also characterized by mass spectrometry and N-terminal sequence analysis of the minor C-terminal metabolite ($SNAP\text{-}25_{198\text{–}206}$) following purification by reverse-phase HPLC (Ekong *et al.*, 1997). N-terminal sequence analysis was performed by Dr D. Pappin of the Imperial Cancer Research Fund, Lincoln's Inn Field, London.

Assessment of BoNT/A-induced proteolytic cleavage of recombinant SNAP-25 by ELISA. An enzyme-immunoassay was developed to assess the endopeptidase activity of BoNT/A towards the recombinant substrates SNAP-25-MBP and r-$SNAP\text{-}25_{134\text{–}206}$. Wells of microtitre plates were coated with 2 μg substrate ml^{-1} in coating buffer (0·05 M $NaCO_3$, pH 9·6) for 16 h at 4 °C. Non-specific adsorbtion sites were blocked for 1 h at 37 °C with 5 % skimmed milk in PBST. Plates were then incubated with serial dilutions of the reduced purified neurotoxin in 50 mM HEPES buffer containing 2 mM DTT and 20 μM $ZnCl_2$ at 37 °C. After washing, BoNT/A-cleaved SNAP-25 was detected by incubation with 5 μg R2 primary antibody ml^{-1}, followed by a 1/2000 dilution of goat anti-rabbit IgG conjugated to HRP (90 min incubation each at 37 °C). Dilutions of detection antibodies were made in PBST containing 2·5 % skimmed milk. Finally, the substrate solution containing 0·5 mg 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ml^{-1} (ABTS) and 0·04 % H_2O_2 in 0·05 M citric acid, pH 4·0, was added. Colour was allowed to develop at room temperature for 30 min and $A_{4\cdot5}$ was measured.

To optimize the microtitre-based endopeptidase assay, the influence of time, pH, buffers, ionic strength, temperature and potential inhibitors on the activity of BoNT/A was examined. Wells of microtitre plates were sensitized with SNAP-25-MBP and incubated under different conditions with serial dilutions of reduced purified BoNT/A (initial dilution 0·5 nM). BoNT/A-cleaved SNAP-25 was detected as described above and the extent of cleavage was expressed as a percentage of the control value obtained with 50 mM HEPES, pH 7·0, containing 2 mM DTT and 20 μM $ZnCl_2$.

Measurement of the endopeptidase activity of therapeutic BoNT/A. The endopeptidase activity of therapeutic preparations of BoNT/A was determined using the optimized enzyme-immunoassay. Therapeutic preparations of BoNT/A were pre-reduced by incubating one vial with 0·5 ml 50 mM HEPES, pH 7·0, containing 10 mM DTT and 20 μM $ZnCl_2$ (37 °C for 30 min). This was diluted to 2 mM DTT in the reaction buffer

and added in serial dilutions to wells of microtitre plates previously sensitized with SNAP-25-MBP for 60 min at 37 °C. BoNT/A-cleaved SNAP-25-MBP was detected immunologically as described above and the extent of cleavage was expressed in relation to the cleavage detected with a reference preparation comprising an in-house standard of BoNT/A preparation.

Bioassay. The potency estimates of therapeutic preparations of BoNT/A were performed using the methods described by Sesardic *et al.* (1996) and McLellan *et al.* (1996) for routine control of this product and were all expressed as activity against an in-house standard used as a reference.

RESULTS

Expression and purification of fusion protein and recombinant SNAP-25 fragment

Cytoplasmic extracts from induced cultures were found to contain larger amounts of a 49 kDa protein than extracts from uninduced cultures (data not shown). The size of the protein band was consistent with the fusion of MBP and the SNAP-$25_{134-206}$ fragment (molecular masses of 42 and 7 kDa, respectively). Maximum expression of fusion protein was observed 24 h after induction and was estimated to constitute about 10–20 % of total soluble cellular extract as measured by densitometry (UVP gel documentation package SW2000, Cambridge).

Cytoplasmic extracts from *E. coli* harbouring the SNAP-25 gene product were fractionated by chromatography on an amylose affinity column. The eluate consisted of 30–40 % fusion protein (the rest being free MBP) (Fig. 2). To prepare SNAP-$25_{134-206}$, the column eluate was treated with Factor Xa and the SNAP-25 fragment purified by ultrafiltration through a Centricon filter. The fragment, which was not readily stained with colloidal gold, was visible on immunoblots only with the R3 antibody, specific for the intact SNAP-25 (Fig. 2). Steps in the purification and the yields of protein are shown in Table 1. Typically, 55–80 mg MBP/SNAP-25-MBP (with SNAP-25-MBP purity of 30–40 %) and 2–4·5 mg SNAP-$25_{134-206}$ (purity 100 %) was obtained from cell lysates containing about 900 mg total protein.

Production of antibodies specific for intact and BoNT/A-cleaved SNAP-25

Rabbit polyclonal antiserum (R1, R2 and R3) to peptides SNAP-25_{1-14}, SNAP-$25_{190-197}$ and SNAP-$25_{193-206}$, were prepared and antibody titres were examined by ELISA using antigen-coated plates. Antibody titres ranged from

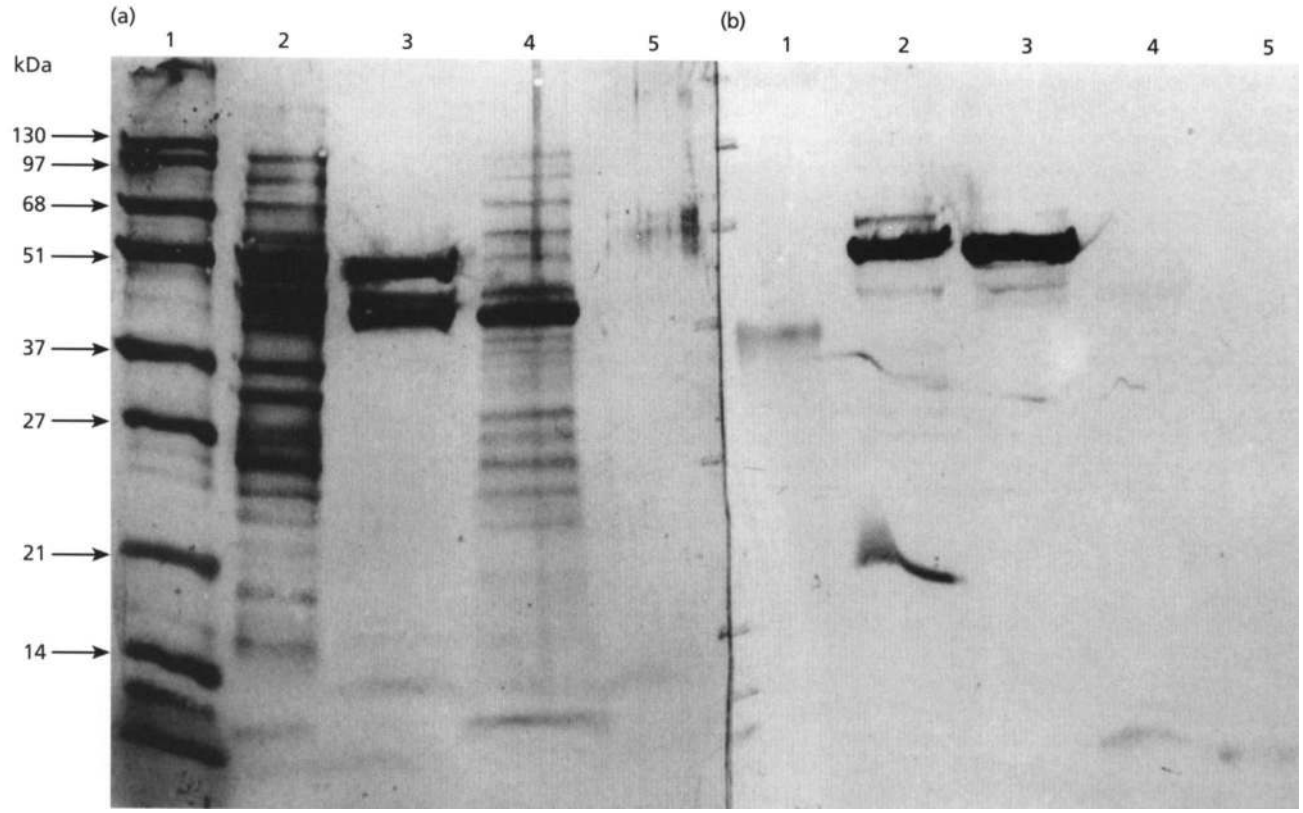

Fig. 2. Purification of recombinant SNAP-25-MBP and SNAP-$25_{134-206}$. (a) Colloidal gold-stained SDS-PAGE and (b) Western blot using anti-SNAP-$25_{190-197}$ (R2) antibody of: lane 1, molecular mass markers; 2, crude lysate from trypsinized *E. coli*; 3, amylose column eluate containing a mixture of fusion protein and free MBP; 4, a mixture of products of Factor Xa cleavage; 5, purified SNAP-25 fragment. SDS-PAGE was carried out in 10–20 % gradient gels under reducing conditions.

Table 1. Purification of fusion protein and recombinant SNAP-25 fragment

Step	Total protein (mg)	Yield (mg)	Purity after SDS-PAGE (%)
E. coli cell lysate	598	100	< 5
SNAP-25-MBP	100	10·2	30–40
SNAP-25 fragment	2·5	0·42	100

Table 2. ELISA titres of anti-SNAP-25 antipeptide antibodies

Antibody	SNAP-25 sequence	Specificity	Titre ($\mu g\ ml^{-1}$)†
R1 (serum)	1–14	SNAP-25 (N-terminal)	40
R1 (AP-IgG)	1–14	SNAP-25 (N-terminal)	1·70
R2 (serum)	190–197	BoNT/A cleavage product of SNAP-25 (N-terminal)	4·70
R2 (AP-IgG)	190–197	BoNT/A cleavage product of SNAP-25 (N-terminal)	0·20
R3 (serum)	193–206	SNAP-25 (C-terminal)	1·10
R3 (AP-IgG)	193–206	SNAP-25 (C-terminal)	0·08

* AP-IgG, Affinity-purified IgG.

† Antibody concentration at an A_{405} of 1·0.

8–40 μg protein ml^{-1} for the crude antiserum to 0·08–1·1 μg protein ml^{-1} for affinity purified IgG (Table 2). Specificity of antibodies was also examined by Western blotting. The R1 antibody recognized a band of about 27 kDa in crude synaptosomal preparations, corresponding to native SNAP-25, but as expected it did not recognize the recombinant fusion protein or fragment (data not shown). The R3 antibody, however, recognized both the native SNAP-25 in crude synaptosomes and the recombinant SNAP-25 substrates (SNAP-25-MBP and r-SNAP-$25_{134–206}$) (Fig. 2), with apparent molecular masses of 50 and 8 kDa, respectively. The R2 antibody was not reactive with native or recombinant SNAP-25 substrates in samples which had not been treated with toxin, but recognized a major band with a molecular mass similar to that of the parent substrate after treatment with type A toxin (shown in Fig. 3 for SNAP-25-MBP). The specificity and titres of the antibodies to SNAP-25 are as summarized in Table 2.

Characterization of the endopeptidase activity of BoNT/A

The proteolytic cleavage of native (data not shown) and recombinant SNAP-25 by purified BoNT/A was assessed by Western blotting. Native SNAP-25 in crude synaptosomes (permeabilized with 1% *n*-octyl-β-D-glucopyranoside) and the recombinant SNAP-25 substrates were cleaved by purified BoNT/A with loss of reactivity to the R3 antibody, specific for intact substrate, and a concomitant gain in reactivity to the R2 antibody, specific for cleaved substrate (Fig. 3). These results indicate that BoNT/A cleaves recombinant SNAP-25, resulting in the formation of an epitope (SNAP-$25_{190–197}$) which is recognized by the R2 antibody. Confirmation that the specific peptide bond Gln_{197}-Arg_{198} was cleaved by BoNT/A in the SNAP-$25_{134–206}$ fragment was achieved by N-terminal sequence analysis and electrospray mass spectroscopy of the C-terminal SNAP-$25_{198–206}$ metabolite isolated by reverse-phase HPLC, giving the expected sequence and mass of 920·1 Da (extrapolated 920·7 Da) (data not shown).

The influence of a number of different conditions on the endopeptidase activity of purified BoNT/A towards SNAP-25-MBP was examined using microtitre plates sensitized with this reagent. The time course of cleavage by BoNT/A was typical of an enzyme reaction: there was an increase in the rate of cleavage which was linear initially, but decreased later due to depletion of substrate and/or accumulation of product (data not shown). This increase in the initial rate of cleavage was inhibited by 75% on the inclusion of 2 mM EDTA in the cleavage buffer. There was an absolute requirement for reduction of toxin and no cleavage was observed if reducing agent (10 mM DTT or 20 mM β-mercaptoethanol) was omitted from buffers. Although cleavage was observed when reducing agent was added at the beginning of the reaction without prior reduction of toxin, this was preceded by a lag period (10–40 min depending on the reducing agent and its concentration). This lag period was eliminated by treating toxin with reducing agent prior to cleavage reaction. The optimum time for toxin pre-reduction with 10 mM DTT was 30 min at 37 °C with 70% of maximum observed cleavage rate determined at time 0 and 35% determined at 2 h.

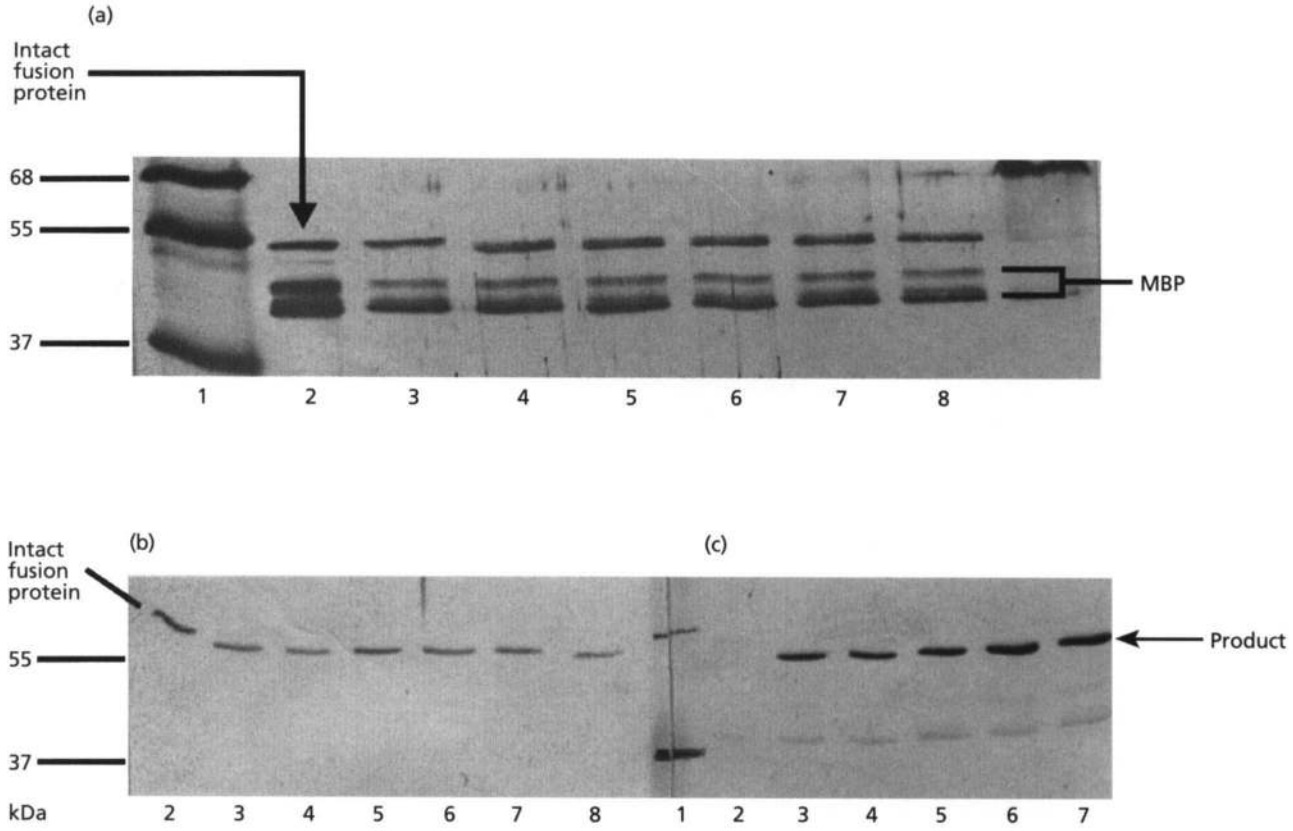

Fig. 3. Time course for proteolytic cleavage of recombinant SNAP-25 by purified BoNT/A. SDS-PAGE and immunoblotting of the recombinant SNAP-25 were performed following incubation with purified BoNT/A for up to 240 min. SDS-PAGE was carried out on 12% gels followed by transfer to nitrocellulose membrane and (a) staining with colloidal gold, (b) immunoblotting with R3 antibody specific for intact SNAP-25 ($SNAP\text{-}25_{193\text{–}206}$) and (c) immunoblotting with R2 antibody ($SNAP\text{-}25_{190\text{–}197}$) specific for one of the BoNT/A cleavage products. Lanes: 1, molecular mass markers; 2, intact fusion protein ($t = 0$); 3–8, SNAP-25-MBP treated with purified BoNT/A toxin for 15, 30, 60, 120, 180 and 240 min, respectively.

Table 3. Effect of buffers on the cleavage of SNAP-25-MBP by BoNT/A

Wells of microtitre plates were sensitized with SNAP-25-MBP (2 μg ml^{-1}) and treated with serial dilutions of reduced purified BoNT/A (initial dilution = 0·013 nM) diluted in different buffers, pH 7·0, with 2 mM DTT and 20 μM $ZnCl_2$. Values represent the mean activity ± SD for six independent determinations expressed as a percentage of activity in 50 mM HEPES buffer, pH 7·0, with 2 mM DTT and 20 μM $ZnCl_2$.

Buffer	Concentration (mM)	Cleavage of SNAP-25-MBP (percentage of control activity)
HEPES	50	100
HEPES	100	85 ± 8·9
HEPES	200	45 ± 10·2
Triethanolamine	50	59 ± 6·1
Tris/HCl	50	47 ± 8·5
Sodium phosphate	50	6 ± 3·4

Following toxin reduction, the presence of low levels of reducing agent in the reaction buffer (2–5 mM) was required to maintain high rates of cleavage. Higher initial rates (two- to fourfold) of cleavage were observed when DTT was used rather than β-mercaptoethanol.

The cleavage of SNAP-25-MBP by BoNT/A was influenced by the type and concentration of buffer used in the reaction. Table 3 shows the rates of reaction with various buffers at pH 7·0. Rates of cleavage observed with triethanolamine, Tris/HCl and, in particular,

sodium phosphate buffers, were lower than those observed with HEPES buffers. Increasing the concentration of HEPES buffer from 50 to 20 mM had a marked inhibitory effect on the cleavage rate.

Optimal endopeptidase activity of BoNT/A towards the substrate was observed at pH 7·0; there was a rapid decrease in the rate of cleavage on either side of this pH. The endopeptidase activity of BoNT/A was also strongly inhibited by an increase in the ionic strength of the buffer. An increase in the concentration of NaCl from 0 to 50 mM and to 100 mM resulted in a decrease of 60 and 87%, respectively. Optimum reactions were considered to be in 50 mM HEPES buffer, pH 7·0, containing 2 mM DTT and 20 μM $ZnCl_2$ without sodium chloride (data not shown).

The temperature profile for BoNT/A proteolytic activity was bell-shaped, with an optimum at 37 °C; there was no reaction at 0 °C and the rate of the reaction decreased rapidly beyond 37 °C (data not shown). Preincubation of reduced purified toxin (0·013 nM) for 30 and 120 min at 37 °C prior to initiation of reaction resulted in a 40 and 85% loss of activity, respectively.

Effect of inhibitors

The effect of a number of inhibitors on the endopeptidase activity of BoNT/A was assessed. For these reactions, reduced toxin was pre-incubated for 30 min at 37 °C with twice the concentration of potential inhibitor required in the final reaction mixture. Wells of substrate-coated plates were then treated for different periods of time with the toxin/inhibitor mixture at 37 °C. The results are presented in Table 4. High concentrations of metal ion chelators such as EDTA (5 mM) or 1,10-*o*-phenanthroline (1·2 mM) and divalent cations such as Zn^{2+} (0·5 mM) completely inhibited the activity of BoNT/A, while the reducing agent DTT, which has weak metal-chelating activity, was inhibitory above 10 mM. This profile confirms the zinc-dependent metalloprotease activity of BoNT/A towards the recombinant substrate.

Assessment of the proteolytic activity of therapeutic BoNT/A

The enzyme-immunoassay employing microtitre plates sensitized with SNAP-25-MBP or SNAP-$25_{134-206}$ was used to estimate the endopeptidase activity of therapeutic preparations of BoNT/A. Titration curves for three different therapeutic preparations, including an in-house reference standard, showed good dose-response profiles (Fig. 4a) in the dilution range equivalent to 1·0–31·6 mouse LD_{50} ml^{-1}. The minimum level of toxin activity that could be detected ranged from 0·1 to 0·8 mouse LD_{50} ml^{-1} ($n = 16$). Different therapeutic preparations of BoNT/A were compared for their relative activities determined by the endopeptidase assay and the *in vivo* bioassay. There was a very good correlation between toxin activity detected by the two systems employing either SNAP-25-MBP ($r = 0.95$, $n = 23$, slope = 1.03; Fig. 4b) or SNAP-$25_{134-206}$ ($r = 0.96$, $n = 16$, slope = 1; data not shown).

DISCUSSION

The fact that clostridial neurotoxins are highly specific zinc-endopeptidases which selectively target different components of the synaptic vesicle/synaptic membrane docking system offers an attractive basis for the development of *in vitro* assays for toxin detection. As BoNT/A selectively targets SNAP-25, at position 197–198, we expressed a SNAP-25 fragment (sequence 134–206) incorporating this cleavage site as a fusion protein with MBP in *E. coli* using the pMAL-c2 expression vector which facilitated subsequent purification of the recombinant protein (SNAP-25-MBP) by amylose-based affinity chromatography. The yield of protein eluting from the amylose column was high (up to 60 mg in some preparations) and consisted of approximately 40% free MBP which was in agreement with observations noted by other workers (Li *et al.*, 1994; Fukuoka *et al.*, 1993). Most importantly, the fusion protein of SNAP-25-MBP was effectively cleaved by BoNT/A and the presence of free MBP did not apparently interfere with endopeptidase activity. Good yields of purified SNAP-$25_{134-206}$ peptide [0·9–2·1 mg (l culture medium)$^{-1}$] could be obtained by cleavage of the fusion protein with Factor Xa and further purification by ultracentrifugation. Based on these results, the pMAL-c2 system is suitable for the preparation of recombinant SNAP-25 for use in the development of an endopeptidase assay for the *in vitro* detection of BoNT/A activity.

Recombinant fusion protein SNAP-25-MBP and SNAP-$25_{134-206}$ peptide were both efficiently cleaved by purified BoNT/A and by this toxin in therapeutic formulations. That BoNT/A was able to cleave recombinant SNAP-25 at position Gln_{197}-Arg_{198} was confirmed by Western blotting using an antibody targeted to an epitope on SNAP-25 which is created only after cleavage with the type A toxin (190–197).Whereas anti-peptide antibody to SNAP-25_{1-14} reacted with both intact and BoNT/A-cleaved SNAP-25, antibody to SNAP-$25_{190-197}$ reacted selectively with the BoNT/A-cleaved SNAP-25 peptide, making it a selective detecting reagent in the microtitre-based endopeptidase assay described here.

Further confirmation that BoNT/A cleaved recombinant SNAP-25 at position Gln_{197}-Arg_{198} was obtained by mass spectrometric and N-terminal sequence analysis of the putative 9 aa product of the reaction (Ekong *et al.*, 1997) and was in agreement with other studies (Schiavo *et al.*, 1993a, b).

A microtitre-based endopeptidase assay for BoNT/A activity was developed using the recombinant substrate and targeted antibodies. A large number of assay conditions utilizing both purified BoNT/A and therapeutic formulations were investigated. These experiments confirmed that the toxin needed to be reduced, either with DTT or β-mercaptoethanol, for effective

Table 4. Effect of inhibitors on the cleavage of recombinant SNAP-25 by BoNT/A

Reduced purified BoNT/A (0·013 nM) was incubated for 3 min at 37 °C with twice the concentration of inhibitor required in the final reaction made up in HEPES buffer, pH 7·0, 2 mM DTT and 20 μM $ZnCl_2$. Serial dilutions of toxin/inhibitor mixture were used to treat wells of a SNAP-25-MBP-sensitized microtitre plate for 60 min at 37 °C. Activity was expressed as a percentage of that in the absence of inhibitors and represents the mean of two independent determinations.

Inhibitor	Concentration (mM)	Cleavage of r-SNAP-25 (percentage of control)
None	–	100
DTT	10	105
	50	61·0
EDTA	2	9·1
	5	0
Captopril	2	96·6
	10	23·3
	15	4·5
o-Phenanthroline	0·13	26·7
	0·50	3·5
Phosphoramidon	2	110
Zn^{2+}	0·20	98
	0·50	2·1

endopeptidase activity. These results suggest that only the two-chain form of the toxin, in which the inter-chain disulphide bond has been reduced, has appreciable proteolytic activity, as is the case for other clostridial neurotoxins (Schiavo *et al.*, 1993b; Shone & Roberts, 1994; Foran *et al.*, 1994; Hallis *et al.*, 1996) and as shown recently for BoNT/A (Schmidt & Bostian, 1995). In agreement with the observations of Schmidt & Bostian (1995), increasing the concentration of DTT from optimum (10 mM) to 50 mM inhibited 61% of activity, due most likely to the weak metal chelating activity of DTT. Similarly, consistent with the inhibitory profiles of zinc-dependent endopeptidases (Schiavo *et al.*, 1992, 1993a) Zn^{2+} and metal-ion chelators such as EDTA inhibited >90% of the proteolytic activity of BoNT/A at 2 mM and 500 μM respectively. The effects of recognized inhibitors of metallopeptidases were also studied. Whereas *o*-phenanthroline inhibited >90% activity at 500 μM, phosphoramidon and captopril were less inhibitory, with virtually no loss of activity at 2 mM, in agreement with their low inhibitory effect against the peptidase activity of tetanus light chain on its natural substrate, VAMP (Soleilhac *et al.*, 1996).

The endopeptidase activity of purified BoNT/A and this toxin in therapeutic formulations showed a bell-shaped pH profile, with an optimum at pH 7·0 for both preparations. Since co-ordination of a zinc molecule by histidine residues within the active site of the toxin enzyme is thought to be crucial for catalytic activity (Schiavo *et al.*, 1992, 1993a), the reduced activity observed at pH 6·0 could reflect changes in the ionization state of these histidine moieties. At the other end of the spectrum, the reduction in activity above pH 7·5 most likely reflects decreased stability of the toxin apoprotein which is known to be labile above pH 7·3 (Schantz & Johnson, 1992).

The use of synthetic peptides as substrates in *in vitro* assays for clostridial neurotoxins has recently been addressed by several workers. A fluorescence-based assay for determination of tetanus toxin peptidase activity using HPLC was described by Soleilhac *et al.* (1996) and HPLC was also used to characterize botulinum type B (Wictome *et al.*, 1996) and botulinum type A (Schmidt & Bostian, 1995; Ekong *et al.*, 1997) endopeptidase activity *in vitro*. These assays have been useful in confirming the kinetics and optimum conditions for the endopeptidase cleavage reaction. In particular, such assays have confirmed the number and identity of the critical residues necessary for substrate design. However, such assays are less useful in the rapid laboratory detection of toxin and at present do not provide the sensitivity required for detection of samples with low toxin activity such as clinical formulations. Thus, the assay for type A toxin described by Schmidt & Bostian (1995) requires 5–30 μg pure type A toxin ml^{-1} in the reaction.

A microtitre-based endopeptidase assay for type A toxin has been recently described by Hallis *et al.* (1996) using a synthetic peptide of $SNAP\text{-}25_{137\text{–}206}$, comparable in size with the recombinant peptide described here. The detection limit for purified type A toxin was described as $1{\cdot}3 \pm 0{\cdot}25$ ng ml^{-1}, equivalent to approximately 200 mouse LD_{50} ml^{-1} for this toxin. The enzymic amplification of the assay increased the sensitivity tenfold (Hallis *et al.*, 1996). Even without an amplification system we were able to detect the equivalent of 0·2–1·0

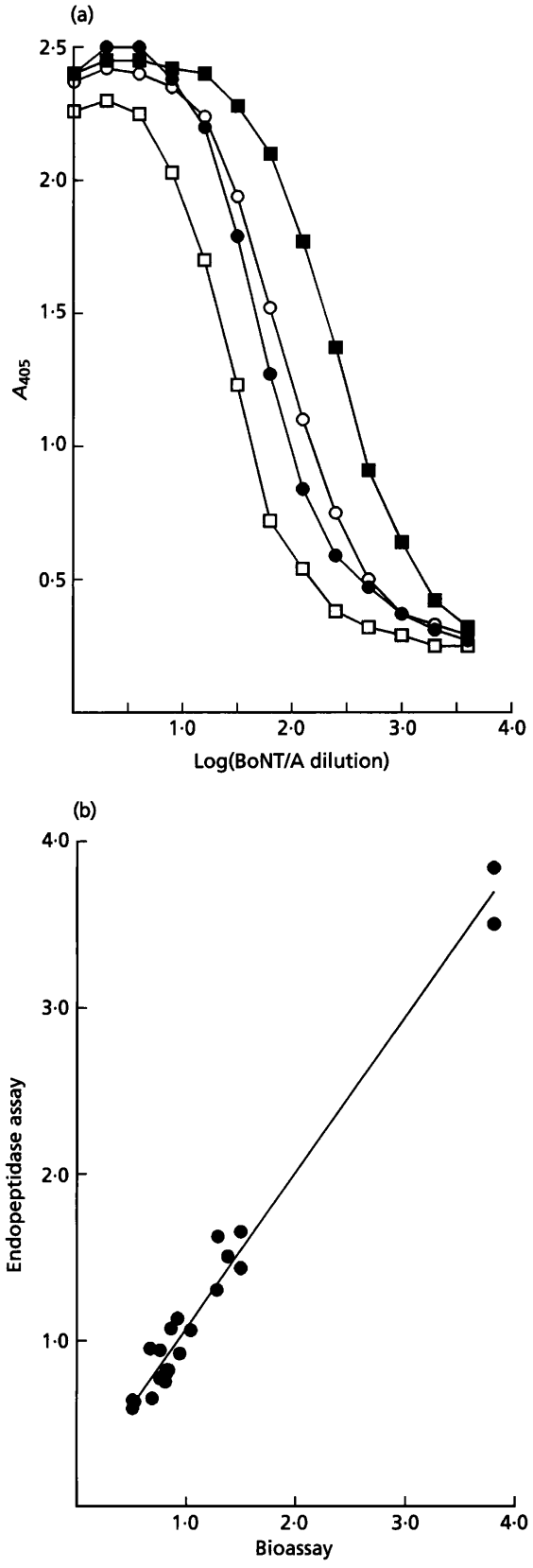

Fig. 4. Endopeptidase activity of therapeutic preparations of BoNT/A. (a) Microtitre plates sensitized with SNAP-25-MBP were treated with serial dilutions (initial dilution = 2 ml per vial) of three different therapeutic preparations of BoNT/A (□, 1×; ○, 2×; ■, 3×; ●, in-house reference standard) which had all previously been reduced as described in the text and incubated for 2 h at 37 °C. Cleaved SNAP-25-MBP was detected using the R2 antibody specific for the cleavage product as described in the Methods. (b) Comparison of the relative potency of 23 different therapeutic preparations of BoNT/A determined using the endopeptidase assay and *in vivo* bioassays. The regression line ($r = 0.95$) had a slope of 1·03.

mouse LD_{50} ml^{-1} for BoNT/A in purified and clinical preparations, making this the most sensitive *in vitro* assay for detection of biologically active BoNT/A.

As the major factor determining the sensitivity of such an assay is the rate of cleavage of the synthetic peptide (Hallis *et al.*, 1996) it is possible that the conditions used in this presentation are more favourable. It is unlikely that the amount of peptide bound to the wells of microtitre plates will affect the rate of cleavage reaction. This was confirmed by the similar activity profiles obtained for SNAP-25-MBP, containing up to 40% free MBP, and purified peptide of $SNAP\text{-}25_{134\text{–}206}$. The optimum buffer for toxin activity was similar to that reported by Hallis *et al.* (1996) but preparation of substrate, its binding to the microtitre plates via Cys residues, higher concentration of DTT in the reaction and use of 1% foetal serum albumin were different. Although we did confirm that inclusion of serum albumin at concentrations of 1–5 mg ml^{-1} can favour the endopeptidase reaction, higher concentrations can be inhibitory. In agreement with Schmidt & Bostian (1995) we have also confirmed that increasing the molarity of HEPES buffer and substituting HEPES buffer with triethanolamine, Tris or phosphate will all substantially decrease the catalytic activity of the toxin. Many factors have been shown to influence the rate of cleavage of the synthetic peptide by the toxin and these will all determine the sensitivity of detection in endopeptidase assays, but it is also possible that the assay sensitivity was increased by the use of a longer epitope (8 aa) for detecting antibody and its affinity purification.

Another critical factor in determining the sensitivity and specificity of endopeptidase assays is the size of the peptide used as a substrate. A number of studies of the substrate specificity of clostridia neurotoxins suggest that the full-length target proteins are cleaved more efficiently than shorter peptide fragments containing the toxin cleavage sites (Soleilhac *et al.*, 1996; Foran *et al.*, 1994; Shone & Roberts, 1994). Schmidt & Bostian (1995) reported detection of higher activity for $SNAP\text{-}25_{187\text{–}203}$ than for the $SNAP\text{-}25_{167\text{–}206}$ peptide in proteolysis by purified BoNT/A in their HPLC assay. However, the K_m for the best substrate was determined as 5·0 mM, which is some 10^7 times higher than that determined by other workers for this activity (Niemann *et al.*, 1996). The same authors further showed the $SNAP\text{-}25_{146\text{–}206}$ peptide to have only 33% of activity of the full length SNAP-25. Our own studies with the full length $SNAP\text{-}25_{1\text{–}206}$ and $SNAP\text{-}25_{137\text{–}206}$ peptide con-

firmed that these substrates were cleaved by BoNT/A and that the full length protein is the better substrate (Ekong *et al.*, 1997). Although our studies confirmed that the 72-mer peptides used here may not be the optimum for such assays, the high sensitivity achieved with the recombinant peptide described here indicates that there was a good cleavage reaction under the conditions employed and that the sensitivity of the assay was comparable with or better than for the bioassay currently used.

One of the advantages of the *in vitro* assay described here is its specificity which is defined by the specific cleavage of the target protein by the toxin coupled with specific immunodetection of the cleavage product. Thus *Clostridium botulinum* toxin type E, which also cleaves SNAP-25 at position Arg_{180}-Ile_{181} (Schiavo *et al.*, 1993a), would remain undetected by antibodies selective for type A toxin detection. Potential problems due to cross-reactivity with other clostridial neurotoxins should be minimal. However, the presence of other neurotoxins may well interfere with the sensitivity due to the presence of a 9-residue motif, which serves as a common recognition site for all clostridial neurotoxins (Rossetto *et al.*, 1994). This will have to be determined. Furthermore, the observation that *Clostridium botulinum* type C toxin also cleaves SNAP-25 (Foran *et al.*, 1996; Williamson *et al.*, 1996) at a position close to that cleaved by BoNT/A (Dr O. Dolly, personal communication) has also opened the possibility of interference in mixed preparations. However, the fact that type C toxin cleaves SNAP-25 at a rate 2000-fold lower than that of type A toxin (Niemann *et al.*, 1996) suggests this may not present a problem.

In this study we have described a simple microtitre-based assay which utilizes a recombinant fragment of SNAP-25, spanning the BoNT/A toxin cleavage site as substrate, together with targeted anti-peptide antibodies for measuring BoNT/A endopeptidase activity *in vitro*. The assay is very sensitive, technically simple and rapid and could therefore be used to screen the activities of a large number of samples containing active type A toxin. With a sensitivity greater than that of the mouse bioassay, this assay represents the most sensitive *in vitro* technique to date for the detection of biologically active BoNT/A toxin. The assay has been shown to be particularly suitable for the detection of BoNT/A in clinical preparations where its use would eliminate the use of large numbers of animals currently required for testing purposes. Clinical preparations containing type A toxin, although not necessarily pure, do not contain other neurotoxin serotypes and are free from protease activities which may compromise the sensitivity of such assays for clinical and poisoning incident specimens. Although the endopeptidase assay described here has advantages over previous *in vitro* assays for this toxin, a potential limitation of this approach is that no assessment of receptor-binding and internalization activities of the toxin is made. In addition, the assay takes no account of the possible role of phosphorylation on toxin activity (Ferrer-Montiel *et al.*, 1996). It has yet to be determined whether these limitations are likely to compromise the usefulness of the assay to replace the *in vivo* bioassay procedures. If so, the presence of the receptor binding domain on the toxin could be confirmed by immunodetection with specific antibodies and the phosphorylation status of the toxin could be evaluated as necessary. Clearly, further validation of the endopeptidase assay for toxin detection and its application for monitoring clinical preparations and biological samples is required.

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