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Development of in vitro assays for the detection of botulinum toxins in foods

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Abstract

Currently the only accepted method for the detection of botulinum neurotoxin in contaminated samples is the mouse bioassay. Although highly sensitive this test has a number of drawbacks: it is expensive to perform, lacks specificity and involves the use of animals. With increasing resistance to such animal tests there is a need to replace the bioassay with a reliable in vitro test. Over the past six years it has been demonstrated that all the botulinum neurotoxins act intracellularly as highly specific zinc endoproteases, cleaving proteins involved in the control of secretion of neurotransmitters. In the work described, this enzymatic activity has been utilised in assay formats for the detection in foods of neurotoxin of the serotypes involved in food-borne outbreaks in man. These assays have been shown to have a greater sensitivity, speed and specificity than the mouse bioassay. It is envisaged that such assays will prove realistic alternatives to animal-based tests.

Keywords: *Clostridium botulinum*; Toxin detection; Food safety

1. Introduction

Various strains of the bacterium *Clostridium botulinum* produce a family of seven structurally related but antigenically different protein neurotoxins (types A to G) [1]. Serotypes A, B, E and F are associated with the often fatal syndrome botulism, in man. Much effort has been imparted by the food industry to ensure that food treatment processes prevent the growth and toxin production of *C. botulinum* and there is a need for rapid, sensitive and specific assays for these toxins. At present the only method of confidence for the detection of the toxins is the acute toxicity test performed in mice [2]. Whilst this test is exquisitely sensitive, with a detection limit of 10–20 pg of neurotoxin ml^{-1} for the most active toxin serotypes, it has a number of drawbacks: it is expensive to perform, requires a large number of animals and is not specific for the neurotoxin serotype. In addition the test takes up to 4 days to complete. The increasing resistance to such animal tests has also required the development of alternative rapid

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in vitro assays with the sensitivity and the reliability of the mouse bioassay. A number of immunoassay systems have been reported with sensitivities comparable to the mouse bioassay [3,4]. These methods, however, require complicated, expensive amplification systems which have not become widely available. In addition these immunoassays do not measure the biological activity of the neurotoxin and can lead to false positive results.

The botulinum neurotoxins have been demonstrated to act at the cellular level as highly specific zinc endoproteases cleaving various isoforms of three neuronal proteins controlling the docking of the synaptic vesicles with the synaptic membrane. Neurotoxin from serotypes A and E cleave specifically the 25 kDa synaptosomal associated protein (SNAP-25) [5], whilst neurotoxin from serotypes B and F act on a different intracellular target, vesicle-associated membrane protein (VAMP) or synaptobrevin [6,7]. Studies have shown that synthetic peptides of VAMP are able to be cleaved by the type B neurotoxin [8,9]. These peptides have been exploited in the development of in vitro assays based on the cleavage of solid-phase immobilised peptide substrates, by the type B neurotoxin, and their detection using highly specific antibody reagents [10]. Whilst such assays are rapid, specific and incorporate a measurement of the biological activity of the neurotoxin, they do not match the sensitivity of the mouse bioassay to be realistic replacements. In addition, the stringent conditions required to support the endopeptidase activity of the neurotoxins is unlikely to be supported in a wide range of food products [9]. Here we describe an assay format for the detection of neurotoxin from proteolytic type B strains, with a sensitivity comparable to the mouse bioassay.

2. Materials and methods

2.1. Preparation of type B neurotoxin

BoNT/B (Okra) was cultured in Cooked Meat Carbohydrate Medium (Oxoid) for 48 h at 37°C. The level of neurotoxin was assessed by sandwich ELISA calibrated against purified type B neurotoxin. The culture supernatants were diluted to 1 ng ml^{-1} (neurotoxin) in 50 mM HEPES, 150 mM NaCl, 1 mg ml^{-1} bovine serum albumin, pH 7.4 and stored at −70°C. The toxicity of this toxin standard was assessed using the mouse bioassay as described previously in [2].

2.2. Preparation of food extracts

Food extracts were prepared following an identical procedure for the mouse bioassay. Food was shaken vigorously with equal volume of gelatin-phosphate buffer and stored at +4°C for 18 h. The sample was then centrifuged at $13\,000 \times g$ for 20 min at 4°C, after which the supernatant was removed and spiked with 1 MLD_{50} ml^{-1} of type B neurotoxin. For the generation of toxin in situ food samples (20 g) were autoclaved in universal bottles and allowed to cool. *C. botulinum* spores were then injected in the centre of the food in 100 μl of distilled water (4.3×10^2 g^{-1} food). The inoculated food samples were incubated anaerobically at 30°C for 4 days after which extracts were prepared as described.

2.3. Synthesis of type B peptide substrate

A peptide substrate representing residues 60 to 94 of human VAMP isoform 1 (VAMP (60–94)) was synthesized, purified and characterised as described in [8]. A C-terminal cysteine was added to the peptide which was post-synthetically modified to incorporate a biotin moiety as described in [11].

2.4. Production of antibodies to peptides

Antisera were raised against the peptide FESSAAKC, which represents the C-terminal side of the cleavage site on VAMP as described in [10].

2.5. Preparation of immunoaffinity columns

Immunoaffinity columns for the extraction of the type B neurotoxin were prepared using cyanogen bromide activated Sepharose 4B (Pharmacia-LKB) following the manufacturer's instructions. One ml of gel slurry was then added to a disposable plastic column (65 mm × 10 mm, Rhône Diagnostics Technologies) giving approximately 100 μl of packed gel, containing 10 μg of immobilised mAb, per column. The columns were stored at +4°C.

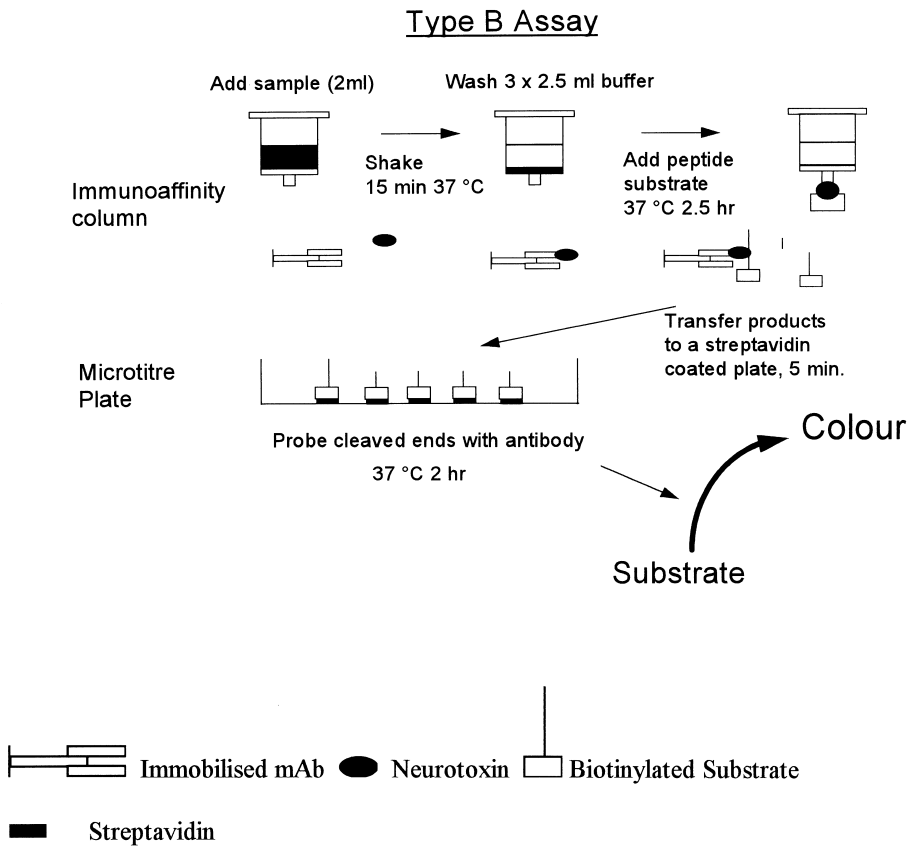

Fig. 1. Type B assay.

2.6. *C. botulinum type B neurotoxin assay*

Storage buffer was drained from the immunoaffinity columns and 2 ml samples were added. The columns were then sealed and shaken horizontally at 37°C for 15 min, after which the columns were washed three times with 2.5 ml of 50 mM HEPES, 20 μM $ZnCl_2$, pH 7.4 (HZ buffer) and the column drained. Hundred μl of 25 μM peptide substrate was added in HZ buffer containing 10 mM dithiothreitol. The columns were then shaken at 37°C for 2 h in an upright position. Four hundred μl of phosphate buffered saline containing 0.1% Tween-20 (PBS-Tw) was then added to each column, mixed and 100 μl samples transferred to a streptavidin coated microtitre plate (Immulon 2-Dynatech). The plate was then shaken for 5 min at 37°C, after which unbound material was removed by washing. Antibody specific to the cleaved peptide was then added (1.2 μg ml^{-1} in PBS-Tw containing 5% foetal calf serum) for 1 h at 37°C. Unbound material was removed by washing, after which rabbit anti-guinea pig immunoglobulin horseradish peroxidase conjugate was added for 1 h at 37°C. After washing tetramethylbenzidine substrate solution was added.

3. Results

An assay system for the detection of type B neurotoxin is depicted in Fig. 1. The toxin was abstracted from the food sample using an immunoaffinity column comprising of two monoclonal antibodies that are specific for proteolytic type B neurotoxin. These mAbs had previously been shown to recognise toxin derived from a wide range of proteolytic type B strains, recognise separate antigenic determinants on the neurotoxin and also do not interfere with

Table 1
Detection of type B neurotoxin in spiked food extracts and neurotoxin generated in situ using the in vitro assay

	Spiked food extracts			In situ generated toxin				
	Pate	Cheese	Cod	Pate	Cheese	Cod	Mince	Sausage
Mean OD	2.26	1.83	2.58	0.56	0.42	2.16	2.66	2.66
% CV	25.5	39.4	11.2	8.9	9.5	14.8	3.4	0.8
n	50	49	54	3	3	3	3	3
Mouse test	1	1	1	neg	neg	>1000	10–100	1–10

Food extracts were spiked with 1 MLD_{50} ml^{-1}. In situ derived toxin was prepared as described in Section 2. In vitro assay data are expressed as mean $OD_{450\,nm}$; *n*, number of tests; % CV, percentage coefficient of variance. Absorbance readings for the negative controls comprising of unspiked food extracts were typically 0.02–0.04 OD units. Values for the mouse test are given in MLD_{50} ml^{-1}; neg, sample was negative in mouse lethality test.

the endopeptidase activity of the neurotoxin on binding, data not shown. Unbound material was then removed by washing and a biotinylated peptide substrate was added. After substrate cleavage the peptide fragments were captured on a streptavidin coated microtitre plate and the cleaved peptide fragments detected using an antibody which was specific to the newly exposed N-terminus of the peptide substrate. Optimum sensitivity of the assay was achieved using 25 μM peptide and cleaving for 2.5 h at 37°C. The detection limit for the assay was found to be approximately 0.5 MLD_{50} ml^{-1}, employing a cut-off of 0.5 absorbance units above background and taking 1 MLD_{50} ml^{-1} to be equal to 10 pg ml^{-1} of neurotoxin, data not shown. The assay was able to detect 1 MLD_{50} ml^{-1} in spiked pate, cod and cheese extracts in approximately 5–6 h as well as in situ derived toxin generated by growth of *C. botulinum* in the food products, Table 1. In addition the assay was found to be more sensitive than the mouse bioassay when the two assays were performed in parallel with identical food samples spiked with crude type B neurotoxin, Table 2.

4. Discussion

In the present study a novel, rapid, in vitro assay has been developed for the detection of type B neurotoxin from proteolytic strains in food products, with a sensitivity greater than the mouse bioassay. Unlike previous solid-phase based assays the assay format described can be used in a range of test media, as the non-bound material that may interfere with the endopeptidase activity of the toxin is removed by washing prior to addition of the peptide substrate. The assay has a far greater sensitivity than previous assays [10], and this result is achieved by presentation of the peptide in solution where it is more efficiently cleaved compared with solid-phase immobilised peptides. The assay was found to be effective in recognising neurotoxin generated in situ, following a procedure similar to the current challenge test performed by the food industry. In vivo analysis of the contaminated pate and cheese samples which generated lower absorbance values in the in vitro assay indicate that this is the result of lower levels of contamination and not due to the in vitro assay under-performing when these food products were tested, Table 1. The assay format described only detects active and not denatured toxin and in this respect the assay more closely reflects the

Table 2
Comparison of the ability of the type B detection assay and the mouse lethality test to detect crude neurotoxin present in spiked food samples.

Toxin (pg ml^{-1})	Pate		Sausage		Buffer	
	Assay	MT	Assay	MT	Assay	MT
50	2.78	+	2.02	+	2.57	+
25	2.80	+	1.90	+	2.54	+
12.5	2.81	+	2.09	+	2.69	+
6.3	2.15	+	1.82	−	1.80	+
3.1	1.24	−	1.19	−	1.09	−
1.5	0.78	−	1.05	−	1.02	−
0	0.02	−	0.004	−	0.04	−

Values for the assay are the mean of four absorbance readings at 450 nm. The mouse lethality test (MT) was performed following AOAC standard method 977.26 using four mice per sample; a positive result was taken if any mice died with botulism-associated symptoms over the 4 days of the test.

mouse bioassay than a conventional ELISA. In addition to date no false positive results have been encountered and this is due to the combined specificity of the capture mAbs and that of the endopeptidase activity of the toxin. The sensitivity of the assay described allows a visual reading and it is predicted that such assays will be used to give a yes/no answer for the presence or absence of toxin in a sample within 5–6 h. A similar approach has resulted in the development of similar assay formats for the detection of neurotoxin from serotypes A, E and F, data not shown. Whilst extensive validation of the assay is required before it will replace the mouse bioassay it is hoped the assay will greatly reduce the number of mice used in the detection of botulinum neurotoxins.

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References

[1] Sakaguchi, G., Ohishi, I. and Kozaki, S. (1986) Botulism: Structure and chemistry of botulinum. In: Handbook of Natural Toxins, Vol. 4: Bacterial Toxins (Hardegree, M.C. and Tu, A.T., Eds.), pp. 191–216. Marcel Dekker, New York.

[2] Kautter, D.A. and Solomon, H.M. (1976) Collaborative study of a method for the detection of *Cl. botulinum* and its toxins in foods. J. Assoc. Anal. Chem. 60, 541–545.

[3] Doellgast, G.J., Triscott, M.X., Beard, G.A., Bottoms, J.D., Cheng, T., Roh, B.H., Roman, M.G., Hall, P.A. and Brown, J.E. (1993) Sensitive enzyme-linked immunosorbent assay for the detection of *Clostridium botulinum* neurotoxins A, B, and E using signal amplification via enzyme-linked coagulation assay. J. Clin. Microbiol. 31, 2402–2409.

[4] Shone, C.C., Wilton-Smith, P., Appleton, N., Hambleton, P., Modi, N.K., Gatley, S. and Melling, J. (1985) Monoclonal antibody-based immunoassay for type A *Clostridium botulinum* neurotoxin is comparable to the mouse bioassay. Appl. Environ. Microbiol. 50, 63–67.

[5] Schiavo, G., Santucci, A., DasGupta, B.R., Mehta, P.P., Jontes, J., Benfenati, F., Wilson, M.C. and Montecucco, C. (1993) Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. FEBS Lett. 335, 99–103.

[6] Schiavo, G., Benefenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B.R. and Montecucco, C. (1992) Tetanus and botulinum-B neurotoxins block transmitter release by proteolytic cleavage of synaptobrevin. Nature 359, 832–835.

[7] Yamasaki, S., Baumeister, A., Binz, T., Blasi, J., Link, E., Cornille, F., Rogues, B., Fykse, E.M., Sudof, T.C., Jahn, R. and Niemann, H. (1994) Cleavage of members of the synaptobrevin/VAMP family by types D and F botulinum neurotoxins and tetanus toxin. J. Biol. Chem. 269, 12764–12772.

[8] Shone, C.C., Quinn, C.P., Wait, R., Hallis, B., Fooks, S.G. and Hambleton, P. (1993) Proteolytic cleavage of synthetic fragments of VAMP isoform-2 by botulinum type B neurotoxin. Eur. J. Biochem. 217, 965–971.

[9] Shone, C.C. and Roberts, A.K. (1994) Peptide substrate specificity and properties of the zinc-endopeptidase activity of botulinum type B neurotoxin. Eur. J. Biochem. 225, 263–270.

[10] Hallis, B., James, B.A.F. and Shone, C.C. (1996) Development of novel assays for botulinum type A and B neurotoxins based on their endopeptidase activities. J. Clin. Microbiol. 34, 1934–1938.

[11] Bayer, A.E., Zalis, M.G. and Wilchek, M. (1986) 3-(N-Maleimido-propionyl) Biocytin: a versatile thiol-specific biotinylating reagent. Anal. Biochem. 149, 529–536.