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Release of proteolytic activity following reduction in therapeutic human serum albumin containing products: Detection with a new neoepitope endopeptidase immunoassay

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ABSTRACT

Botulinum type A toxin (BoNT/A) is defined by its specific endopeptidase cleavage of SNAP25 between Gln^{197} and Arg^{198} under reducing conditions. The neurotoxin is widely used for therapeutic or cosmetic purposes, but should not contain other toxin serotypes or unwanted protease activities. Using a neoepitope endopeptidase immunoassay, additional cleavage between Arg^{198} and Ala^{199} was detected with a range of therapeutic BoNT/A products confirming an earlier report of an unidentified proteolytic component. By developing the assay and making it insensitive to BoNT/C1, any activity due to the type C1 toxin was excluded. Therapeutic preparations consist of ng quantities of toxin protein which are typically stabilised by 0.125–30 mg of HSA. An excellent correlation ($R^2 = 0.993$) between HSA content per vial and measured activity was obtained within the therapeutic BoNT/A products tested. No activity was detected in any of the non-albumin formulated preparations, thereby identifying HSA as the source of the unknown protease for the first time. To investigate the cause of this activity, either as an intrinsic molecular activity of albumin or due to an albumin-associated purification contaminant, further studies on a variety of commercial plasma-derived HSA products or recombinant HSA materials free from potential plasma contaminants were carried out. The measured proteolytic levels were highly consistent amongst preparations, and could all be partially inhibited by the presence of zinc and blocked by PKSI-527 and aprotinin. By contrast, the data did not support the role of plasmin, kallikrein, trypsin, α_2-antiplasmin–plasmin complexes or HSA purification contaminants, PKA (prekallikrein activator) or kallikrein-like activity. Taken together, these findings indicate a new intrinsic proteolytic activity of the albumin molecule revealed under reducing conditions as the source of the unexpected Arg–Ala cleaving activity.

1. Introduction

Low doses of botulinum toxin are widely used therapeutically to locally paralyse specific muscles for clinical or cosmetic benefit. The purified toxin is now considered the treatment of choice for disorders such as blephrospasm, strabismus and dystonia [1]. Botulinum type A toxin (BoNT/A) is the most widely used therapeutic serotype, and these products are highly regulated to ensure their consistency and safety in the clinical setting. Furthermore, new retargeted botulinum toxin LHn fragments are being developed to target pain, respiratory diseases, neuroendocrine cancer and obesity, which also require consistency testing [2].

The light chain component of botulinum toxins is a zinc dependent metalloprotease that specifically cleaves soluble SNARE proteins following reduction within the nerve terminal [3–5]. A single amino acid separates the $SNAP25^{1–206}$ (synaptosomal-associated protein 25) cleavage points of type A (between Gln^{197} and Arg^{198}) and C1 (between Arg^{198} and Ala^{199}) toxins [6]. Despite these similarities, a new highly specific neoepitope endopeptidase immunoassay has recently been described for botulinum type C1 toxin (BoNT/C1) [7]. As well as not recognising the intact substrate, the neoepitope detecting antibody has outstanding specificity and the BoNT/C1 cleavage site antibody (anti-$SNAP25^{198}$) fails to cross-react or recognise the BoNT/A cleavage product $SNAP25^{1–197}$ [7]. Likewise, type A cleavage site-specific antibody (anti-$SNAP25^{197}$)

Abbreviations: HSA, human serum albumin; BSA, bovine serum albumin; PKA, prekallikrein activator; BoNT/C1, botulinum type C1 toxin; BoNT/A, botulinum type A toxin; DTT, DL-dithiothreitol.

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fails to recognise the C1 cleaved peptide SNAP25$^{1-198}$ [8]. Using fluorescently labelled SNAP25 and reverse phase chromatography separation, Hunt et al. [9] detected an unexpected cleavage product in a therapeutic botulinum type A toxin, corresponding to cleavage between Arg^{198} and Ala^{199} by either trypsin, a trypsin-like enzyme or BoNT/C1; however, they were unable to conclusively identify its source. These findings led them to speculate the presence of either damaged BoNT/A or a contaminating protease due to poor processing or storage [10].

The current study describes the development and novel application of a highly specific neoepitope endopeptidase immunoassay to detect a new proteolytic activity released under reducing conditions and consistently found in a range of BoNT/A therapeutic products containing HSA as an excipient. For this purpose, an anti-SNAP25^{198} assay insensitive to BoNT/C1 whilst more sensitive for the novel protease activity was developed and the source of this activity, either as an intrinsic albumin molecule activity or albumin purification contaminant, investigated.

2. Materials and methods

2.1. Test samples

Frozen aliquots of control purified BoNT/A, complex free (Hall strain) and BoNT/C1 haemagglutinin complex (Brazil strain) at 20,000 mouse LD_{50}/mL in gelatine (2 mg/mL) phosphate (50 mM disodium hydrogen orthophosphate) buffer pH 6.5 (GPB) were used as indicated. A range of freeze-dried therapeutic BoNT/A products from six manufacturers from around the world were donated for use plus a in-house toxin (A/23 containing 0.5 mg HSA/vial [11]). A range of seven anonymously labelled therapeutic HSA liquid preparations (batches #1 and #2 from manufacturer A, batches #3 and #4 from manufacturer B and batches #5–#7 from other manufacturers) were diluted as required in the experiments from their 200 or 250 mg/mL formulations. A highly purified BSA powder (A3059, ~99% pure by electrophoresis), Cellastim™ recombinant human serum albumin (rHSA, A9731, expressed in rice ≥90%), Albucult™ rHSA (A6608, expressed in *Saccharomyces cerevisiae* and supplied as a 100 mg/mL solution ≥99%), trypsin (T-8642, from bovine pancreas, TPCK treated), kallikrein (K1004, 2.4 U/mg), and plasmin purified from human plasma (P1867, 3.8 U/mg) were all purchased from Sigma–Aldrich Ltd. (Dorset, UK). CellPrime™ AF-S rHSA at 100 mg/mL was purchased from Novozymes Ltd. (9501-20, expressed in *S. cerevisiae*, Nottingham, UK).

2.2. SNAP25 peptide substrate

A 70 a.a. SNAP25$^{137-206}$ peptide (VTNDA-RENEM-DENLE-QVSGI-IGNLR-HMALD-MGNEI-DTQNR-QIDRI-MEKAD-SNKTR-IDEAN-Q*RA*TK-MLGSG) was synthesised as previously described [8].

2.3. Primary detecting antibodies

SNAP25$^{191-198}$ with a cysteine tag (C-RIDEANQR) was custom synthesised by Immune Systems Ltd. (Paignton, UK) and coupled to KLH via the cysteine tag. Antibodies to the peptide were then raised in sheep (MicroPharm Ltd./Ig-Innovations Ltd., Llandysul, Wales, UK) to provide SNAP25^{198} neoepitope-specific antibodies. Serum taken at 18 weeks post primary immunisation was subsequently used throughout in these studies without further purification [7].

2.4. Neoepitope endopeptidase immunoassay under reducing conditions

Polystyrene 96 well plates (Nunc Maxisorp, VWR International Ltd., Leicestershire, UK) were coated with 100 μL/well of 3 μg/mL SNAP25$^{137-206}$ substrate in 50 mM carbonate buffer (pH 9.6) overnight at room temperature (RT), decanted and blocked with 300 μL/well of 50 mg/mL skimmed milk powder in phosphate buffered saline, 0.05% (v/v) Tween 20 (PBST) for 90 min at RT. Plates were then washed three times with distilled water and dried. These could then be stored for up to 3 months with a dry desiccator capsule at −20 °C. Test samples were suitably diluted in either reaction buffer A (50 mM HEPES, 20 μM $ZnCl_2$, pH 7.0, 0.5% (v/v) Tween 20, 5 mM DTT), or B (buffer A containing 86 mM NaCl), or C (buffer B without zinc), as indicated and serial dilutions (either 1 in 2 or 1 in 1.278) performed on a substrate-coated plate in reaction buffer. Plates were then individually sealed and incubated at 37 °C (without stacking) for 18 h. These were then washed three times with PBST, blot dried, and 100 μL/well of neoepitope-specific detecting antibody (sheep anti-SNAP25^{198} at a 1 in 800 dilution) in antibody buffer (25 mg/mL skimmed milk powder in PBST) added, sealed and incubated at RT for 90 min without stacking. Plates were washed again and 100 μL/well of rabbit anti-sheep-HRP (31480, Perbio Science UK Ltd., Northumberland, UK, 1 in 4000 dilution in antibody buffer) added and plates incubated as before. After washing, 100 μL/well substrate solution (50 mM citric acid pH 4.0, 0.91 mM 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 min. Following development, the plates were briefly shaken and absorbance read at 405 nm using a suitable plate reader.

2.5. Comparison of albumin-associated proteolytic activity in BoNT/A and HSA products under reducing conditions

Using aliquots of HSA (#5) as an internal reference, various batches of BoNT/A or HSA products were diluted and tested in either reaction buffer B or C. Six replicates were performed for both test and reference, on a single substrate-coated plate and the relative activity expressed following probit parallel line analysis [12]. For the sake of clarity an arbitrary value of 100 proteolytic Units/mL (0.5 U/mg HSA) was assigned to the internal reference HSA (#5) in reaction buffer C.

2.6. Enzyme inhibitors

Enzyme inhibitor studies were typically performed in reaction buffer C unless otherwise indicated. The plasma kallikrein inhibitor PKSI-527 (trans-4-aminomethylcyclohexanecarbonyl-phenylalanyl-4-carboxymethylanilide) was purchased from Enzo Life Sciences UK Ltd. (PI-147, Exeter, UK). Aprotinin (A1153, 3900–10,400 KIU/mg purified from bovine lung), α_2-macroglobulin (M6159) and α_2-antiplasmin (A8849, 9.8 U/mg) were purchased from Sigma–Aldrich Ltd. (Dorset, UK). Enzyme–inhibitor complex formation was optimised in the absence of DTT and the following proportions used: 400 μg/mL α_2-macroglobulin/6 μg/mL plasmin mixed and incubated for 2 h at 37 °C, or 30 μg/mL α_2-antiplasmin/15 μg/mL plasmin mixed and incubated for 2 h at RT.

2.7. Measurement of contaminating PKA activity using a chromogenic substrate under non-reducing conditions

Prekallikrein activator (PKA), otherwise known as factor XIIa, or activated Hagman factor, was assayed using the European Pharmacopoeia method by its activation of added plasma prekallikrein [13,14]. The liberated kallikrein activity subsequently being determined by its ability to cleave a specific chromogenic substrate (H-D-Pro-Phe-Arg-pNA.2HCl, S2302, Quadratech Ltd.,

Epsom, Surrey, UK) and measuring the colour change. Test samples and reference (2nd International Standard for PKA, 02/168, at 29 IU/ampoule, obtained from NIBSC, UK) were diluted in buffer (0.05 M Tris–HCl, 0.02 M NaCl, pH 8.0) and tested in replicate as previously described [15]. The relative amount of PKA in each test sample was then calculated by parallel line analysis [12].

2.8. Contaminating kallikrein-like protease activity measured using the neoepitope immunoassay in the absence of DTT

To determine the contribution of contaminating proteases cleaving between Arg^{198} and Ala^{199} under non-reducing conditions the endopeptidase immunoassays were performed as described above (see Section 2.4) except utilising reaction buffer D (buffer C without DTT).

3. Results

3.1. BoNT/C1-like activity detected within therapeutic botulinum type A toxin products

A variable and unexpected BoNT/C1-like activity was found with the therapeutic BoNT/A products containing different amounts of HSA (Fig. 1) using a previously described neoepitope endopeptidase immunoassay developed for measuring BoNT/C1 activity [7]. This activity was not directly related to the products BoNT/A activity as a control non-therapeutic albumin-free formulation of purified BoNT/A expressed no activity.

3.2. Neoepitope endopeptidase immunoassay optimisation

Effects of sodium chloride on the proteolytic activities under reducing conditions were investigated. An increasing sodium chloride concentration in the presence of zinc was shown to progressively inhibit the sensitivity of the assay to BoNT/C1, with no activity detected at concentrations of 86 mM sodium chloride and above (Fig. 2a). This represents a greater than 100-fold decrease in sensitivity between buffer A and B. A similar inhibitory effect was also seen with trypsin activity (30 pg/mL, data not shown). By contrast, a 5–6-fold increase in HSA-associated activity was achieved with 86 mM sodium chloride (Fig. 2b). Therefore, in all subsequent experiments a sodium chloride concentration of 86 mM was utilised to give a more specific and sensitive assay for the albumin-associated activity.

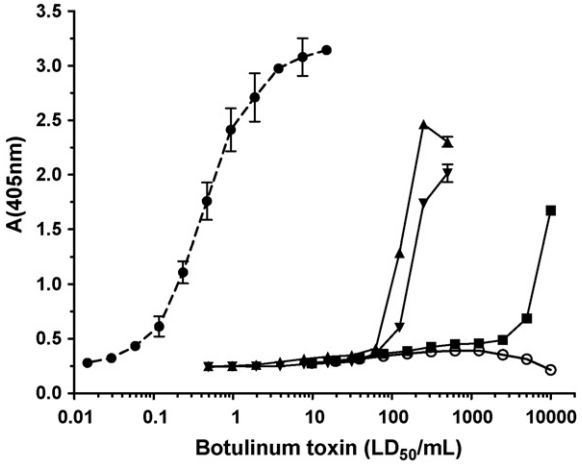

Fig. 1. Use of an established neoepitope endopeptidase immunoassay to measure an unexpected BoNT/C1-like activity within therapeutic botulinum type A toxin products. Assay performed under reducing conditions in buffer A. Therapeutic BoNT/A from three manufacturers with formulations containing different amounts of HSA (solid lines: triangle 0.5 mg/vial; upside-down triangle 1 mg/vial; square 0.125 mg/vial). Positive control BoNT/C1 (broken line) and control BoNT/A (solid line, open circles). Controls were free from albumin. Mean (range, $n = 2$).

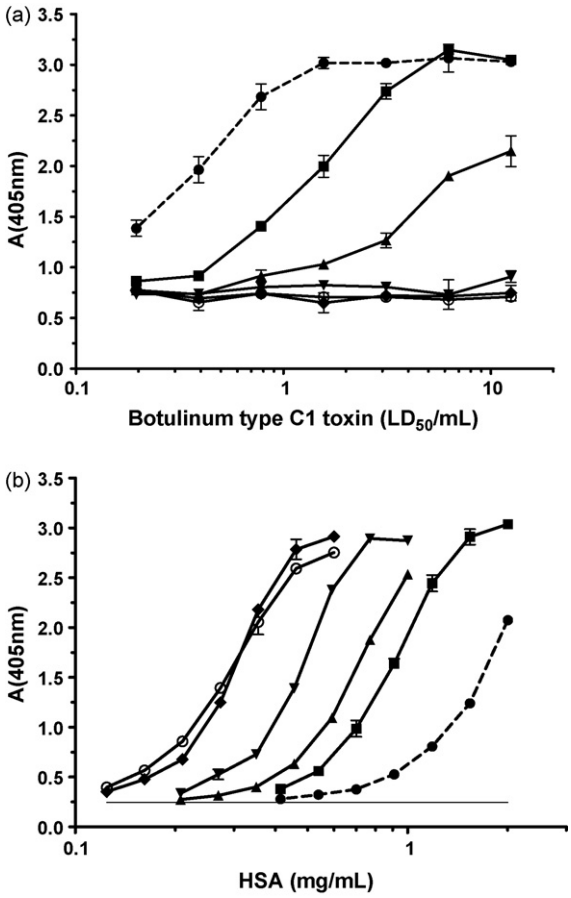

Fig. 2. Effect of sodium chloride concentration on proteolytic activity under reducing conditions. The inhibitory actions of sodium chloride in reaction buffer A seen with BoNT/C1 (a), and contrasting potentiating effect with HSA-associated activity (b), within the neoeptitope endopeptidase immunoassay. Sodium chloride concentrations: 0 mM (solid circle, broken line), 9 mM (square), 17 mM (triangle), 34 mM (upside-down triangle), 86 mM (diamond), 171 mM (open circle) mean (range, $n = 2$).

3.3. Limit of detection

A 3-fold increase in activity was obtained in the absence of zinc (buffer C), applying these optimal conditions (buffer C), a limit of detection of 20 μg/mL (or 0.002%) was obtained with HSA (#5). The cut-off for the limit of detection being determined by the mean negative control ($n = 24$), plus three times the standard deviation.

3.4. Correlation between albumin content in therapeutic BoNT/A products and measured activity under reducing conditions

The new modified neoepitope immunoassay allowed the consistent detection of HSA-associated proteolytic activity in all the BoNT/A products which contained HSA as an excipient. No activity could be detected in either the single therapeutic product which did not contain HSA as a stabiliser (data not shown) or other albumin-free toxin preparations. The internal reference HSA (#5) had an assigned value of 100 proteolytic Units/mL in buffer C, with a linear range between 0.09 and 0.25 mg/mL necessitating the use of serial dilutions as low as 1 in 1.278. Due to the decreased activity and non-parallel dose–response curve of the reference with zinc containing buffer B, the titre corresponding to 50% proteolytic activity

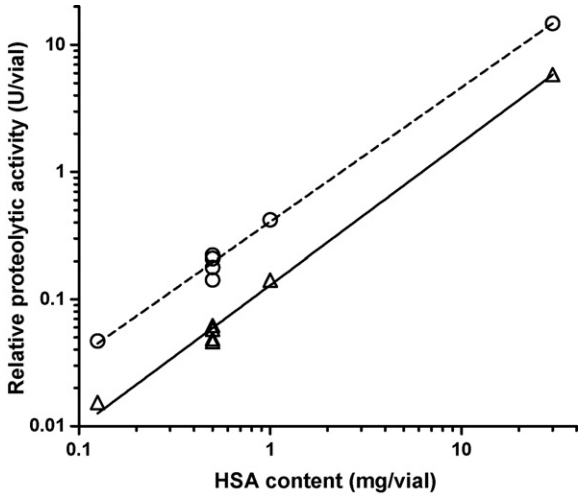

Fig. 3. Correlation between albumin content in dried BoNT/A products and the relative proteolytic activity per vial under reducing conditions. The new modified neoepitope immunoassay performed in the absence (broken line, circle, buffer C) or presence (solid line, triangle, buffer B) of zinc. Six BoNT/A therapeutic products containing HSA were tested with two batches of one product, and the in-house toxin (A/23). Activities were expressed relative to HSA product (#5) taken as the reference with assigned values of 100 or 32.3 Units/mL in buffer C or B, respectively.

of HSA (#5) was read-off and a value of 32.3 proteolytic Units/mL in the presence of zinc (buffer B) assigned. A linear dose–response range was obtained in buffer B between 0.22 and 0.43 mg/mL again necessitating the use of 1 in 1.278 serial dilutions. Relative proteolytic activity was then expressed following parallel line analysis against the respective reference value for HSA (#5). In the presence (buffer B) or absence of zinc (buffer C) 95% confidence limits were less than or equal to ±4.1% or ±6.0% of the determined values, respectively.

When BoNT/A products were tested, both in the presence (R^2 = 0.993) and absence (R^2 = 0.993) of zinc (buffer B or C), excellent correlations between the measured relative activities and known HSA concentrations were obtained, together with consistently lower activities in the presence of zinc (Fig. 3). This data confirmed albumin as the source of this proteolytic activity within BoNT/A products.

3.5. Consistency of the proteolytic activities within a range of plasma-derived liquid therapeutic HSA products under reducing conditions

Using the optimum neoepitope immunoassay conditions the new HSA-associated proteolytic activities were measured. As shown in Table 1, a highly consistent proteolytic activity was measured for all the HSA products (#1–#7) tested. In the presence (buffer B) or absence of zinc (buffer C) 95% confidence limits were less than or equal to ±3.7% or ±4.5% of the determined values, respectively. In the presence of zinc (buffer B) a maximal difference of just 30% was observed between the lowest (#3) and highest (#2) activities with an even smaller difference amongst the other therapeutic samples. However, in the absence of zinc (buffer C) a slightly increased maximal variability (up to 40%) between the lowest (#1) and highest (#6) activities was observed. Again activities were consistently higher in buffer free from zinc. A similar spread of activities was also seen with the range of BoNT/A products (Fig. 3).

3.6. Consistency between recombinant and plasma-purified albumin preparations

A highly conserved activity was obtained with both plasma-purified HSA and recombinant HSA products which were expressed in yeast or rice and therefore free from potential plasma contaminants (see Table 2). Proteolytic activity was observed in both the presence and absence of zinc, although a 3–4-fold decrease was consistently seen in the former case (Table 2). Lower values were obtained with a BSA preparation (data not shown).

Table 1
Consistency of the proteolytic activities within a range of plasma-derived liquid HSA therapeutic products under reducing conditions. The new albumin-specific neoepitope immunoassay performed in the absence (buffer C) or presence (buffer B) of zinc. Activities were calculated by parallel line analysis relative to HSA product (#5) taken as the reference with assigned values of 100 or 32.3 Units/mL in buffer C or B, respectively.

HSA product no.	New albumin-associated proteolytic activity (U/g)	
	Without zinc	With zinc
#1	352	156
#2	467	207
#3	446	147
#4	376	152
#5	500	162
#6	583	167
#7	530	159

3.7. Effect of pH on the new HSA-associated proteolytic activity

Optimal HSA-associated proteolytic activity was obtained at pH 7.0 (pH 6.0 = 28%, pH 6.5 = 79%, pH 7.0 = 100%, pH 7.5 = 62%, and pH 8.0 = 39%) and therefore either characterises the intrinsic molecular activity or eliminates contaminating proteases which work under acidic or basic conditions. Assay performed in reaction buffer C.

3.8. Effects of DTT on proteolytic activity

In order to investigate the possible contribution of a contaminating protease various purified enzymes or unpurified human serum were tested. Using the neoepitope immunoassay, a large decrease in the proteolytic activities of trypsin, plasmin and kallikrein was caused by the presence of DTT (Fig. 4a). By contrast, reducing conditions (buffer C) exposed a partial proteolytic activity from the inert α_2-antiplasmin–plasmin complex (Fig. 4b), but did not have any effect on the α_2-macroglobulin–plasmin complex (data not shown). In the presence of DTT, largely increased proteolytic activities were measured with both human serum and HSA, although a less steep dose–response curve was obtained with the crude serum, which also contains large quantities of non-albumin protein (Fig. 4c). The less steep curve is likely a result of non-albumin-associated proteases with alternative cleavage points.

3.9. Effects of enzyme inhibitors under reducing conditions

A range of enzyme inhibitors were screened against the albumin-associated proteolytic activity to identify an inhibitor and

Table 2
Proteolytic activities of recombinant HSA under reducing conditions. Neoepitope immunoassays performed in the absence (buffer C) or presence (buffer B) of zinc. Relative activities were calculated from the concentrations giving 50% maximal absorbance values.

HSA product	New albumin-associated proteolytic activity (U/g)	
	Without zinc	With zinc
Cellastim™ rHSA	411	116
Albucult™ rHSA	445	142
CellPrime™ rHSA	667	162
Control HSA#5	500	162

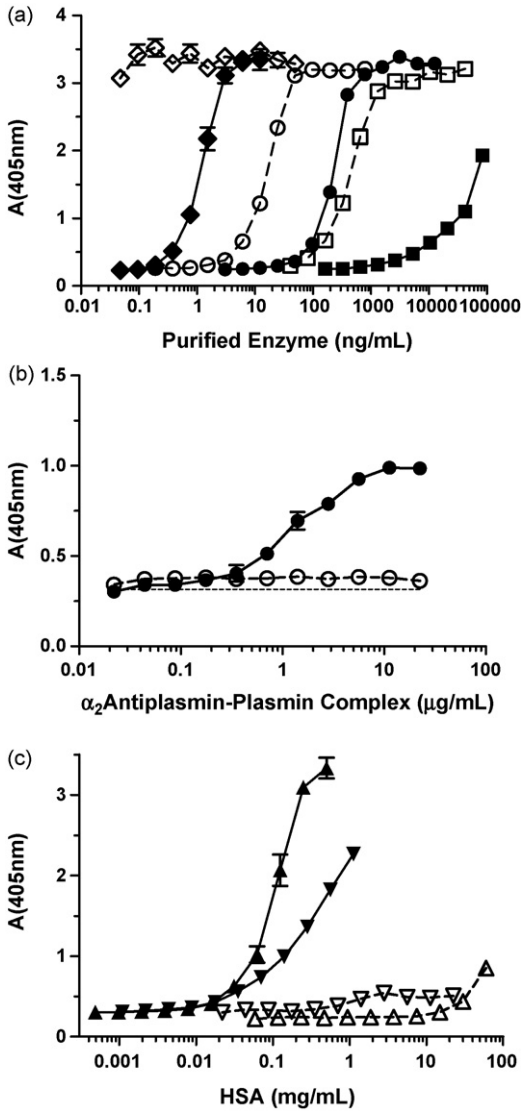

Fig. 4. Effects of DTT on proteolytic activities. The inhibitory effects of reducing conditions on purified enzymes (a), and conversely proteolytic activities exposed under reducing conditions (b and c) with an inhibitor–enzyme complex (b) or serum and HSA (c). Neoepitope immunoassays performed in the presence (solid lines, buffer C) or absence (broken lines, buffer D) of DTT. Purified enzymes (a) trypsin (diamond), plasmin (circle), and kallikrein (square). Blood products (c) HSA#1 (triangle), and human serum (inverted triangle, assuming serum contains 45 mg/mL albumin).

provide evidence as to the source of this activity. Aprotinin (MW 6511 Da) produced a 50% reduction of HSA activity at ~380 nM before a sharp increase in background adversely affected the assay at higher enzyme inhibitor concentrations (data not shown). This inhibitory effect took place despite the presence of reducing conditions which are known to be unfavourable for aprotinin whose full activity depends on the presence of its disulfide bonds [16]. The low molecular weight (474 Da) plasma kallikrein inhibitor PKSI-527 was, however, found to completely inhibit HSA-associated activity at high molar concentrations (data not shown). PKSI-527 produced a 50% inhibition at ~500 μM on a range of albumin materials (HSA, rHSA and BSA). By contrast, a much shallower inhibition curve was obtained with the α_2-antiplasmin–plasmin complex, with a 50% block at approximately 1mM PKSI-527. The assay buffer pH was not adversely affected by either aprotinin or PKSI-527 over the inhibitory range.

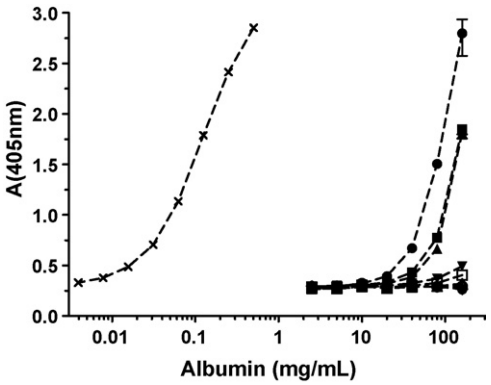

Fig. 5. Contaminating kallikrein-like protease activities of albumin preparations under non-reducing conditions. Neoepitope immunoassays performed in the absence of DTT (buffer D). Albumin preparation: Cellastim™ rHSA (×), HSA#1 (solid circle), HSA#6 (square), HSA#7 (triangle), BSA (upside-down triangle), HSA#4 (open square), HSA#2 (diamond), HSA#3 (open circle), HSA#5 (open triangle), mean (range, $n = 3$). No activity was detected with either CellPrime™ rHSA or Albucult™ rHSA at up to 90 mg/mL (data not shown).

3.10. Contaminating PKA activity under non-reducing conditions

The contaminating PKA activity was measured for plasma-derived liquid therapeutic HSA products (#1–#7) and the following results obtained: 6.0, <2.5, <2.5, 6.0, <2.5, 9.9, <2.5 IU/mL for preparations #1–#7, respectively. No apparent correlation could be found between these PKA values and the modified neoepitope endopeptidase values described in Section 3.5.

3.11. Contaminating kallikrein-like proteolytic activity under non-reducing conditions

Low levels of contaminating kallikrein-like proteolytic activity in the absence of reducing conditions (buffer D) could be clearly detected in HSA products (#1), (#6) and (#7) and was barely detected in both the Sigma "protease free" BSA and HSA product (#4) (Fig. 5). Levels were undetectable in the other products (#2, #3, #5) at albumin concentrations up to 180 mg/mL. An exceptionally high level of kallikrein-like activity was, however, detected with the lower purity Cellastim™ rHSA; this was almost three orders of magnitude higher than the highest HSA preparation (#1). No activity was detected with the purer rHSA preparations CellPrime™ or Albucult™ up to 90 mg/mL, or with a therapeutic grade rHSA (data not shown).

The same order of potency values was also obtained with the therapeutic HSA products when kallikrein activity was determined with the chromogenic substrate (see Section 2.7) without prekallikrein addition (data not shown). No correlations could be found between these kallikrein-like activities and the new HSA-associated proteolytic activity values described in Section 3.5.

4. Discussion

Clinical BoNT/A products are well characterised highly purified materials. They should not contain other neurotoxin serotypes and be free from unwanted protease activities as this could present a concern for batch consistency, stability and safety. An earlier study reported an unidentified proteolytic component in a BoNT/A product which cleaved at the additional Arg^{198}–Ala^{199} position [9]. Using a neoepitope endopeptidase immunoassay, we were able to confirm the previous findings and characterise this unexplained protease activity in a wide range of therapeutic BoNT/A products. Further assay development and modification ruled out any contamination due to BoNT/C1 and provided an assay with

increased sensitivity to this mysterious activity. The uncharacteristically high endopeptidase levels measured in the absence of zinc further distinguished this activity from that of botulinum toxins. Therapeutic botulinum preparations consist of ng quantities of toxin protein which are typically stabilised by mg amounts of HSA. When the BoNT/A therapeutic samples were tested by the modified endopeptidise immunoassay, the specified HSA content per vial gave an excellent correlation with the measured protease levels. No activity was detected in any of the non-albumin formulated BoNT/A preparations, thereby identifying HSA as the source of the unknown protease for the first time. Activity under reducing conditions was measured in all HSA-containing therapeutic products tested (e.g. HSA or BoNT/A products: Botox®, Dysport®, Xeomin® and Neuronox®). The extra Xeomin® cleavage product reported by Hunt et al. [9] under reducing conditions could therefore be explained by its HSA content. This highlights the importance of having sufficiently sensitive and specific botulinum toxin endopeptidase assays for product characterisation, such as the previously reported neoepitope immunoassays [7,8], that allow the HSA or other potentially interfering excipients to be diluted out during *in vitro* testing.

There are two possible sources for this newly described activity: (a) an intrinsic proteolytic activity of the albumin molecule or (b) an albumin purification contaminant. To investigate this hypothesis, a wide range of albumin products, including commercial plasma-derived albumin and recombinant HSA free from potential plasma contaminants, were tested under various conditions. The strongest evidence for an intrinsic molecular mechanism was obtained from the highly consistent activities measured amongst HSA and rHSA products with varying purities. Zinc was found to have a consistent ~3- to 4-fold inhibitory effect on all the HSA and rHSA activities tested, again indicating a highly conserved cause. Lower activity levels were, however, found with a purified BSA preparation, a result that could be explained by a species difference between the bovine and human albumin molecules [17]. We have also identified two enzyme inhibitors PKSI-527 and aprotinin that block the newly discovered HSA activity. Full inactivation was attained with the enzyme inhibitor PKSI-527 using HSA, rHSA and BSA preparations; this again highlighted the similarities. An inherent esterase-like activity in HSA has previously been described using p-nitrophenyl ester substrates under non-reducing conditions [18–20]; this is, however, distinct from a true proteolytic activity capable of cleaving large polypeptide substrates.

Pasteurised albumin solutions prepared from fractionated plasma are known to be contaminated with low levels of plasma impurities which may include prekallikrein activator (PKA), its product kallikrein, and/or possible traces of other proteolytic enzymes [15,21–26]. PKA has a very specific cleavage site between Arg–Ile and would, therefore, not be directly detected by the neoepitope immunoassay [27]; this was confirmed by the absence of any correlation with the new exposed proteolytic activities in the HSA products. The Arg–Ala substrate cleavage point recognised by our neoepitope antibody is, however, shared by various enzymes, including plasma kallikrein, tissue kallikreins, plasmin, factor XIa, and trypsin. In direct contrast to that seen with human serum or HSA materials, all the purified enzymes tested (plasmin, kallikrein and trypsin), with the exception of BoNT, paradoxically had less activity under reducing conditions. This ruled-out plasmin, kallikrein and trypsin as contributory factors. On the other hand, when α_2-antiplasmin, a known HSA contaminant [22], formed an inert complex with plasmin, proteolytic activity could be detected under reducing conditions; this indicated the possible involvement of naturally occurring enzyme inhibitor complexes. Despite the partial activity revealed by the α_2-antiplasmin–plasmin complex under reducing conditions, identical contaminants would not be found in yeast- or rice-derived recombinant albumin and is therefore, insufficient to fully explain the HSA activity. It however, remains a remote possibility that other inert serpin-enzyme contaminants also susceptible to DTT activation could be found in all the different albumin preparations from the range of different sources.

Despite many therapeutic HSA products containing undetectable levels of contaminating kallikrein-like activity measured in the absence of reducing conditions, a variable low level was detected in a few of the HSA products by assays utilising either low molecular weight chromogenic or high molecular weight (70 a.a.) SNAP25 substrates. However, these values failed to correlate with the new proteolytic activity exposed under reducing conditions. Interestingly, one rHSA product tested had a much higher kallikrein-like proteolytic activity than any other product. Although the other purer recombinant products had undetectable kallikrein-like proteolytic activity levels, caution should be exercised by manufacturers of therapeutic products in considering switching stabilisers from HSA to rHSA because of the potential for introducing immunogenic non-human contaminating proteins or enzymes. With an extensive track record of high dosage use, human albumin solution prepared from plasma has gained a reputation as an extremely safe product with fewer side-effects than artificial plasma substitutes [21,28]. Plasma-purified HSA is also widely used as an effective BoNT stabiliser under non-reducing conditions; in fact, some freeze-dried products containing HSA as stabiliser are stored at room temperature for the full 3 year shelf life with no loss of activity [29–31].

After weighing up all the evidence, the consistency of our data across a wide range of HSA and rHSA products indicates a new intrinsic proteolytic activity of the albumin molecule in the presence of DTT. The normal physiological relevance of these findings currently remains unclear. However, this may shed some light on the ability of albumin to activate microglia or its presence within the intracellular reducing environment of the developing nervous system [3,32,33]. The sensitivity of the new HSA-associated endopeptidase assay could be further increased (~doubled) using higher 50 mM DTT concentrations, if required (data not shown). Using this assay unknown HSA concentrations could potentially be measured in un-licensed or counterfeit BoNT products or as a novel method of characterising HSA products.

In conclusion, we have confirmed the presence of an unexpected Arg–Ala cleaving proteolytic component within therapeutic BoNT/A product formulations containing HSA as an excipient. Our findings are consistent with and highly suggestive of an intrinsic proteolytic activity of the albumin molecule exposed under reducing conditions. This novel activity is optimal at pH 7.0 in the presence of 86 mM NaCl, 50 mM DTT and absence of zinc.

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References

[1] D.D. Truong, A. Stenner, G. Reichel, Current clinical applications of botulinum toxin, Curr. Pharm. Des. 15 (2009) 3671–3680.
[2] K.A. Foster, E.J. Adams, L. Durose, C.J. Cruttwell, E. Marks, C.C. Shone, J.A. Chaddock, C.L. Cox, C. Heaton, J.M. Sutton, J. Wayne, F.C. Alexander, D.F. Rogers, Re-engineering the target specificity of clostridial neurotoxins—a route to novel therapeutics, Neurotox. Res. 9 (2006) 101–107.
[3] S. Raina, D. Missiakas, Making and breaking disulfide bonds, Annu. Rev. Microbiol. 51 (1997) 179–202.
[4] G. Schiavo, M. Matteoli, C. Montecucco, Neurotoxins affecting neuroexocytosis, Physiol. Rev. 80 (2000) 717–766.
[5] E.P. Feener, W.C. Shen, H.J. Ryser, Cleavage of disulfide bonds in endocytosed macromolecules. A processing not associated with lysosomes or endosomes, J. Biol. Chem. 265 (1990) 18780–18785.

[6] L.C. Williamson, J.L. Halpern, C. Montecucco, J.E. Brown, E.A. Neale, Clostridial neurotoxins and substrate proteolysis in intact neurons: botulinum neurotoxin C acts on synaptosomal-associated protein of 25 kDa, J. Biol. Chem. 271 (1996) 7694–7699.
[7] R.G.A. Jones, Y. Liu, D. Sesardic, New highly specific botulinum type C1 endopeptidase immunoassays utilising SNAP25 or syntaxin substrates, J. Immunol. Methods 343 (2009) 21–27.
[8] R.G.A. Jones, M. Ochiai, Y. Liu, T. Ekong, D. Sesardic, Development of improved SNAP25 endopeptidase immuno-assays for botulinum type A and E toxins, J. Immunol. Methods 329 (2008) 92–101.
[9] T. Hunt, K. Clarke, D. Rupp, G. Shimizu, K. Tam, Secondary cleavage of a fluorescently labeled SNAP-25 substrate by Xeomin drug product, Toxicon 51 (Supplement 1) (2008) 13.
[10] M.F. Brin, Basic and clinical aspects of Botox, Toxicon 54 (2009) 676–682.
[11] D. Sesardic, T. Leung, D.R. Gaines, Role for standards in assays of botulinum toxins: international collaborative study of three preparations of botulinum type A toxin, Biologicals 31 (2003) 265–276.
[12] D.J. Finney, Statistical Method in Biological Assay, Charles Griffin, London, 1978.
[13] T.J. Snape, D. Griffith, L. Vallet, E.D. Wesley, The assay of prekallikrein activator in human blood products, Dev. Biol. Stand. 44 (1979) 115–120.
[14] Prekallikrein Activator, Ph. Eur., Council of Europe, Strasbourg, France, 2008.
[15] C. Longstaff, M.E. Behr-Gross, A. Daas, F. Lackner, An international collaborative study to replace the 1st international standard for prekallikrein activator, Vox Sang. 88 (2005) 143–151.
[16] H. Ahmed, Principles and Reactions of Protein Extraction, Purification, and Characterization, CRC Press, 2004.
[17] N. Tayeh, T. Rungassamy, J.R. Albani, Fluorescence spectral resolution of tryptophan residues in bovine and human serum albumins, J. Pharm. Biomed. Anal. 50 (2009) 107–116.
[18] H. Watanabe, S. Tanase, K. Nakajou, T. Maruyama, U. Kragh-Hansen, M. Otagiri, Role of Arg-410 and Tyr-411 in human serum albumin for ligand binding and esterase-like activity, Biochem. J. 349 (2000) 813–819.
[19] Y. Sakurai, S.F. Ma, H. Watanabe, N. Yamaotsu, S. Hirono, Y. Kurono, U. Kragh-Hansen, M. Otagiri, Esterase-like activity of serum albumin: characterization of its structural chemistry using p-nitrophenyl esters as substrates, Pharm. Res. 21 (2004) 285–292.
[20] J. Cordova, J.D. Ryan, B.B. Boonyaratanakornkit, D.S. Clark, Esterase activity of bovine serum albumin up to 160 degrees C: a new benchmark for biocatalysis, Enzyme Microb. Technol. 42 (2008) 278–283.
[21] H. Zilg, H. Schneider, F.R. Seiler, Molecular aspects of albumin functions: indications for its use in plasma substitution, Dev. Biol. Stand. 48 (1980) 31–42.
[22] R.L. Gundry, Q. Fu, C.A. Jelinek, J.E. Van Eyk, R.J. Cotter, Investigation of an albumin-enriched fraction of human serum and its albuminome, Proteomic. Clin. Appl. 1 (2007) 73–88.
[23] P.J. Turner, I.F. Young, P.B. Marley, R.W. Herrington, P. Schiff, Albumin solutions—their production and quality control, Dev. Biol. Stand. 67 (1987) 119–127.
[24] B.M. Alving, Y. Hojima, J.J. Pisano, B.L. Mason, R.E. Buckingham Jr., M.M. Mozen, J.S. Finlayson, Hypotension associated with prekallikrein activator (Hageman-factor fragments) in plasma protein fraction, New Engl. J. Med. 299 (1978) 66–70.
[25] P. Matejtschuk, C.H. Dash, E.W. Gascoigne, Production of human albumin solution: a continually developing colloid, Br. J. Anaesth. 85 (2000) 887–895.
[26] Y. Che, F.J. Wilson, J. Bertolini, P. Schiff, D.W. Maher, Impact of manufacturing improvements on clinical safety of albumin: Australian pharmacovigilance data for 1988–2005, Crit. Care Resusc. 8 (2006) 334–338.
[27] D.W. Chung, K. Fujikawa, B.A. McMullen, E.W. Davie, Human plasma prekallikrein, a zymogen to a serine protease that contains four tandem repeats, Biochemistry 25 (1986) 2410–2417.
[28] M.E Barron, M.M. Wilkes, R.J. Navickis, A systematic review of the comparative safety of colloids, Arch. Surg. 139 (2004) 552–563.
[29] M.C. Goodnough, E.A. Johnson, Stabilization of botulinum toxin type A during lyophilization, Appl. Environ. Microbiol. 58 (1992) 3426–3428.
[30] S. Grein, G.J. Mander, H.V. Taylor, Xeomin is stable without refrigeration: complexing proteins are not required for stability of botulinum neurotoxin type A preparations, Toxicon 51 (Supplement 1) (2008) 13.
[31] J. Frevert, Xeomin is free from complexing proteins, Toxicon 54 (2009) 697–701.
[32] J. Uriel, J. Trojan, P. Dubouch, A. Pineiro, Intracellular alpha-fetoprotein and albumin in the developing nervous system of the baboon, Pathol. Biol. (Paris) 30 (1982) 79–83.
[33] C. Hooper, D.L. Taylor, J.M. Pocock, Pure albumin is a potent trigger of calcium signalling and proliferation in microglia but not macrophages or astrocytes, J. Neurochem. 92 (2005) 1363–1376.