Recombinant Botulinum neurotoxin serotype A1 (SXN102342): protein engineering and process development

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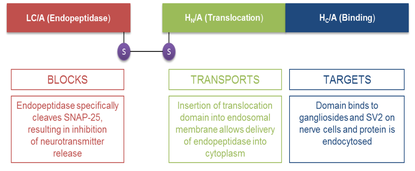
Introduction

Botulinum neurotoxin (BoNT) is effective in the treatment of several movement disorders (Simpson, 2016). To date, over thirty naturally occurring BoNTs have been identified, including eight subtypes of BoNT/A; this natural diversity of BoNTs provides a rich selection of molecules for potential therapeutic exploitation.

Native BoNT/A comprises a family of highly related neurotoxins produced by *Clostridium botulinum* bacteria, all neutralised by the same antisera. BoNT/A1 (subtype A1) is notable, as the Hall-strain BoNT/A used to produce abobotulinumtoxinA (Dysport®), onabotulinumtoxinA (BoTox®) and incobotulinumtoxinA (Xeomin®). An increased understanding of the structure-function relationship of BoNT provides an opportunity to engineer recombinant (r) BoNTs with unique pharmacological properties and therapeutic applications.

rBoNT/A1 (SXN102342) is a multi-domain, multi-functional recombinant protein expressed within *Escherichia coli* (Figure 1). It is composed of a light chain (LC/A) endopeptidase domain, and a heavy chain composed of an N-terminal translocation domain (H_N/A) and a C-terminal neuronal targeting domain (H_C/A).

Figure 1. Schematic of rBoNT/A1 structure and function



LC/A - Light Chain domain of Botulinum neurotoxin serotype A
SNAP-25 - Synaptosomal-associated protein 2 substrate for the LC/A
H_N/A - N-terminal functional domain of the Botulinum neurotoxin serotype A heavy chain
H_C/A - C-terminal functional domain of the Botulinum neurotoxin serotype A heavy chain
SV2 - Synaptic vesicle glycoprotein 2

Methods

BoNT/A sequence alignment

Based on multiple analyses of BoNT/A strains, eight BoNT/A subtypes have been documented. Twenty eight non redundant sequences exist in public databases that can be clearly assigned to the BoNT/A subtype, the UNIPARC accession numbers for these are listed in Figure 2.

The rBoNT/A1 sequences are 1292 – 1297 amino acids in length and 84 – 99% identical to each other. UNIPROT identifier UPI0000001386, Hall-strain BoNT/A used to produce multiple commercial products was identified as the preferred reference sequence. There are notable non-conservative amino acids differences with other sequences.

Results

Here, we describe the construction, expression, purification and characterisation of rBoNT serotype A1 (rBoNT/A1; SXN102342).

Construction of rBoNT/A1

DNA encoding rBoNT/A1 was fully synthesised, codon optimised for soluble expression in *Escherichia coli,* and transformed into a BLR (DE3) research cell bank.

Purification and activation of rBoNT/A1

Batches of cells were cultured in shake flasks at a 1-litre scale. Purification of rBoNT/A1 (SXN102342) was achieved by selective precipitation, followed by hydrophobic interaction and ion exchange column chromatography, coupled with an endoproteinase activation step (Figure 3). The activation stage cleaves the expressed single-chain precursor protein at a specific recognition site and produces the fully active di-chain rBoNT/A1 product, the light and heavy chains being joined by a disulphide bond. Hydrophobic interaction chromatography was then used to separate any residual endoproteinase from the target product.

Figure 2. Botulinum neurotoxin A subtypes

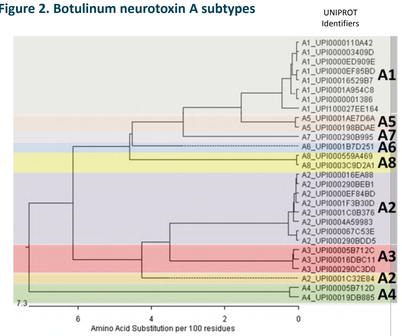
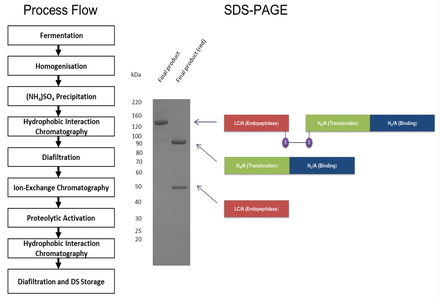


Figure 3. SXN102342 manufacturing process



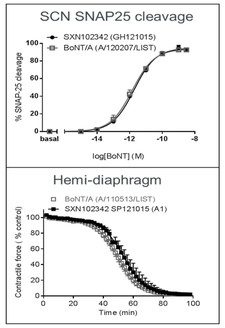
Under non-reducing and denaturing conditions rBoNT/A1 SXN102342 (~150 kDa) maintains the interchain disulphide bond. Under reducing and denaturing conditions SXN102342 is converted into the free light chain (~50 kDa) and the heavy chain (~100 kDa). Each of these moieties has a specific electrophoretic migration pattern.

rBoNT/A1 testing and properties

Batches of rBoNT/A1 (SXN102342) drug substance were characterised against a panel of biochemical and functional assays (Figure 4). Batches of rBoNT/A1 (SXN102342) were more than 95% activated at the endoproteinase activation purification stage. The purification process resulted in final drug substance with greater than 95% purity and endotoxin levels below 2.5 EU/mg. The drug substance was formulated in either phosphate buffered saline (PBS) or PBS supplemented with human serum albumin and sucrose. Native BoNT/A1 from List Laboratories (Campbell, USA) was used as a reference in all assays.

Figure 4. SXN102342 testing and properties

	Mean
Activation (%)	99 ± 0.8
Purity (%)	91 ± 1.4
Endotoxin (EU/mg)	< 2.5
Potency (SCN SNAP25 cleavage, pM)	EC_{50}: 3.1 ± 0.9
Potency (mouse hemi-diaphragm, min)	TP_{50}: 49 ± 3



Cellular and ex-vivo assays

Cellular potency was measured in rat primary embryonic spinal cord neurons (eSCN). SNAP-25 cleavage potency was assessed after treatment of cells with rBoNT/A1 for 24 hours by quantitation of full length and cleaved forms of SNAP-25 by Western blotting.

An *ex vivo* assay was used to measure the effects of BoNT at the neuromuscular junction. *Ex vivo* neuromuscular paralysis was measured by treating preparations of mouse phrenic hemi-diaphragm at a final concentration of 10 pM and measuring time to 50% paralysis upon stimulation.

The resulting rBoNT/A1 product was functionally equivalent to nBoNT/A and offers a possible recombinant alternative to current commercial native BoNT/A1 products.

Conclusions

- Recombinant Botulinum neurotoxin sequences can be readily cloned into industry standard expression constructs for expression by microbial strain.
- Purification of soluble recombinant Botulinum neurotoxins can be achieved using conventional protein chromatography approaches, to a purity in excess of 95%.
- A key aspect of purifying recombinant Botulinum neurotoxins is the requirement for proteolytic activation and conversion from the full-length single-chain precursor to the fully active di-chain product.
- The opportunity to successfully engineer and manufacture rBoNT/A1 acts as a rBoNT reference standard and baseline, for the development of other genetically modified rBoNTs with differentiated pharmacological properties, and new commercial BoNT product opportunities.

Keywords

Botulinum Neurotoxin (BoNT); Serotype A1; Manufacture; Protein engineering; Process development; Product characterisation.

References

Simpson DM, Hallett M, Ashman EJ, et al. Practice guideline update summary: botulinum neurotoxin for the treatment of blepharospasm, cervical dystonia, adult spasticity and headache. *Neurology*.2016;86:1818-1826.

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