An innovative solution to the unique challenges in manufacturing recombinant botulinum neurotoxins

Laura Lovelock [a], Daniel Kwan [a], Kevin Moore [a], Andrew Splevins [a], Philip Marks [b], Andy Hooker [a], Peter Horrocks [a]

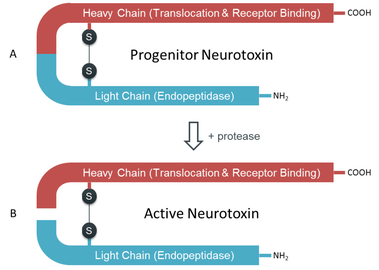
[a]Ipsen Bioinnovation Ltd., 102 Park Drive, Milton Park, Abingdon, Oxfordshire, OX14 4RY, UK
[b]Ipsen Biopharm Ltd., Unit 9 Ash Road, Wrexham Industrial Estate, Wrexham, LL13 9UF, UK

Introduction

The use of recombinant technologies to manufacture botulinum neurotoxins offers significant advantages over traditional production processes, which are carried out by culture of *C. botulinum* bacteria, followed by isolation and purification of the clostridial neurotoxin complex.

Botulinum neurotoxins are initially expressed as single chain polypeptides. In order to be fully active, the single chain form must be converted into a di-chain form, which requires proteolytic cleavage at a site located between the light and heavy chains; the activation loop (Figure 1).

Figure 1. Recombinant toxin activation by proteolysis



Recombinant botulinum neurotoxins are expressed as a single chain polypeptide and can be converted to the fully active di-chain form when incubated with a suitable protease *in vitro*. The protease cleaves the neurotoxin within the activation loop between the light and heavy chains, which remain linked by a disulphide bridge, to produce the di-chain product.

During recombinant manufacturing, *in vitro* activation can be achieved by the addition of a suitable protease during the process. However unwanted proteolytic activity can also occur at sites outside the activation loop before full activation is achieved, resulting in the formation of undesirable truncated products. Solutions to the problem usually require some form of modification of the amino acid sequence; here we present a method for producing full-length, activated recombinant botulinum neurotoxin without the need to introduce sequence modifications.

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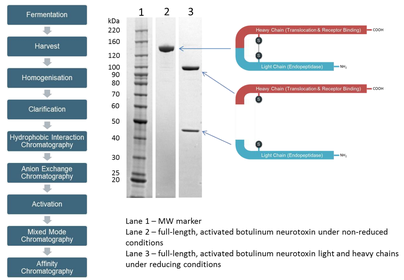
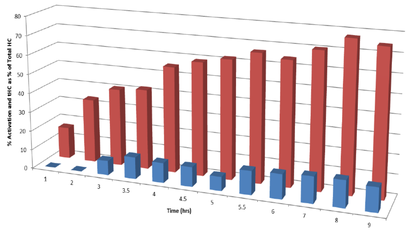
Figure 2. Example recombinant toxin manufacturing process



Lane 1 – MW marker
Lane 2 – full-length, activated botulinum neurotoxin under non-reduced conditions
Lane 3 – full-length, activated botulinum neurotoxin light and heavy chains under reducing conditions

Recombinant botulinum neurotoxins can be manufactured using microbial expression , *in vitro* protease activation and sequential column chromatography to remove host cell and other contaminants to produce a highly purified and fully activated product when analysed by SDS-PAGE under non-reducing and reducing conditions.

Figure 3. Formation of a truncated neurotoxin during proteolytic activation



The formation of truncated neurotoxin (blue bars) occurs early in the activation process and prior to complete conversion of the progenitor neurotoxin into the activated neurotoxin (red bars). In this example, the levels of truncated neurotoxin have reached approximately 20 %, whilst the conversion of the progenitor into the active form is only approximately 80 % complete.

Methods

The recombinant botulinum neurotoxin was expressed as a soluble single polypeptide chain in *Escherichia coli*. After primary recovery steps including centrifugation and mechanical homogenisation, the neurotoxin was purified by sequential column chromatography. During the purification process, the neurotoxin was activated by the addition of a protease, which cleaves the neurotoxin between the light and heavy chains to produce an active, di-chain product, with the light and heavy chains linked by a disulphide bond. Following activation, the neurotoxin is purified further by additional column chromatography steps to remove any remaining host cell contaminants, product related truncates and residual protease (Figure 2).

Optimisation of activation
Activation of the neurotoxin was optimised by screening a range of conditions, including reaction time, temperature, pH, salt and protease concentration. The screens employed both traditional screening cascades and Design of Experiments approaches to identify conditions that maximised the formation of full length, activated di-chain neurotoxin, whilst minimising the presence of any unactivated single chain or truncated neurotoxin products.

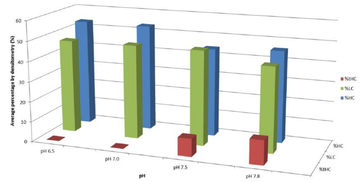
Chromatography resin screening
A range of chromatography resins, including hydrophobic interaction, ion exchange and mixed mode resins were screened for their ability to separate full length, activated di-chain neurotoxin from unactivated single chain and truncated neurotoxin products.

Results

Optimisation of activation
The optimisation of the activation process did not identify any conditions capable of producing only full length, activated neurotoxin, with the absence of any unactivated or truncated neurotoxin.

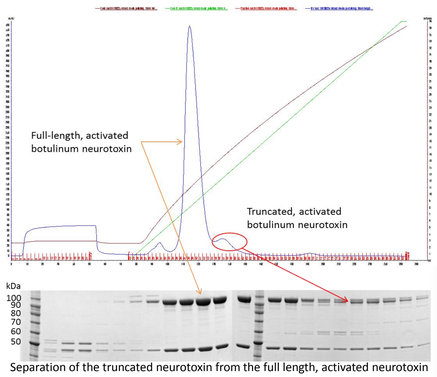
- Figure 3 shows the formation of both full length, activated neurotoxin and a truncated neurotoxin during the *in vitro* activation process. Formation of truncated products occurred before the activation of the neurotoxin was complete, leaving three product related species to be separated; unactivated, full length activated and truncated neurotoxin.

Figure 4. Effect of pH on toxin activation and truncation



In this example the activation process was performed over a pH range from pH 6.5 – 7.8 and the levels of truncated product (red bars) present, once full conversion of the progenitor toxin had been achieved, were assessed by SDS-PAGE and densitometry. The green and blue bars represent the percentage of cleaved light (LC) and heavy chains (HC). Decreasing pH reduced the amount of truncate formed; below pH 7.0 the level of truncated product present in the samples was below the limit of detection (LOD) of the assay.

Figure 5. Separation of truncated product from full length, activated toxin by mixed-mode chromatography



Separation of the truncated neurotoxin from the full length, activated neurotoxin was achieved in this example using a mixed-mode column chromatography resin and eluting the column over a linear gradient of increasing ionic strength. The separation of the two products was visualised by SDS-PAGE under reducing conditions. The truncation has occurred in the heavy chain (HC) of the neurotoxin.

- Figure 4 shows the effect of pH on toxin activation; decreasing the pH of the reaction also reduced the amount of truncated product formed to below the limit of detection (LOD) of the SDS-PAGE assay. It did not prevent truncation entirely, as was shown later when truncated neurotoxin was separated from full length activated neurotoxin by column chromatography.

Chromatography resin screening
The chromatography resin screening did not identify any resins or conditions that were capable of separating the unactivated neurotoxin pre-cursor from the activated and truncate neurotoxin products. The screen did identify chromatography resins and conditions that were capable of separating the full-length activated and truncated neurotoxin products.

- Figure 5 shows the separation of full length activated and truncated neurotoxin by mixed mode chromatography after an activation step at pH 6.5.

By performing the activation step at pH 6.5 and allowing it to reach full conversion of the unactivated neurotoxin, it was then possible to use column chromatography to remove truncated neurotoxin to produce a highly purified (>95 %), fully activated neurotoxin product without the need for any sequence modifications .

Conclusions

- Recombinant neurotoxin can be manufactured without the need to introduce modifications to the amino acid sequence.
- We have developed an approach that forces the enzymatic cleavage reaction beyond completion and then removes unwanted truncated products by protein chromatography.
- This results in a highly purified, fully activated neurotoxin product that can be manufactured for a variety of applications, including therapeutic use.

Keywords
Botulinum neurotoxin (BoNT); Recombinant; Manufacture; Chromatography; Proteolysis; Activation

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