Large scale production of Botulium neurotoxin type A binding domain and characterization by quartz crystal microbalance biosensor technology

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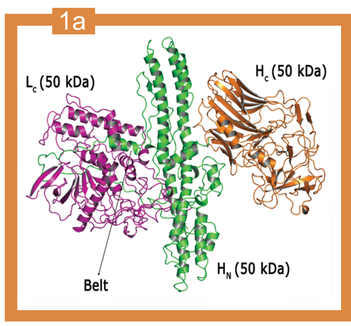
ABSTRACT

Botulinum neurotoxin (BoNT) serotype A is one of the most poisonous toxin that cause the disease botulism for both human and animals. We developed a group of three monoclonal antibodies that can potently neutralize all four known subtypes of BoNT/A. These three antibodies bind non-overlapping epitopes on BoNT/A with high affinity. To support commercial development of the antitoxin drug, large amount of pure BoNT/A domains were essential. Here we report the optimized methods for large scale production of the C-terminal receptor binding domain of the Botulinum toxin A, abbreviated as HCC. High levels of HCC were expressed in E. coli as a recombinant protein and accumulated in the insoluble inclusion body.

A method was optimized to extract HCC from the inclusion body, refold it into active form and purify it using ion exchange chromatography to a purity over 95%. And with AKTA crossflow filtration system, we were able to scale up our highly automated process to a yield of around a hundred milligrams for each purification. To facilitate monitoring active HCC during purification and for better quality control, we also developed a rapid immunoassay that provided real time assay of active HCC using Attana's quartz crystal microbalance sensor technology.

INTRODUCTION

BoNT/A is a protein composed of three functional domains: the C-terminal receptor binding domain (Hc), the translocation domain (Hn) and the N-terminal catalytic domain (Lc). The antitoxin drug being commercially developed for BoNT/A is a combination of three monoclonal antibodies: NX01, NX02 and NX11 which covers non-overlapping epitopes on the toxin domain. NX01 recognizes an unique epitope located at the C-terminal half of the Hc domain (HCC), hence it is a valuable reagent for NX01 development process. It is being used to monitor the stability of NX01 in the combination of the three mAbs, and it is also being used to help determine the pharmacokinetics and pharcodynamics of the three antibodies in pre-clinical animal models. It will be used to monitor NX01 in the following clinical trials as well. So we developed and optimized methods to express large quantities of recombinant HCC in bacteria and to purify HCC in a high throughput manner. We also used quartz crystal microbalance biosensor technology provided by the Attana machine to determine the active HCC in the development process.



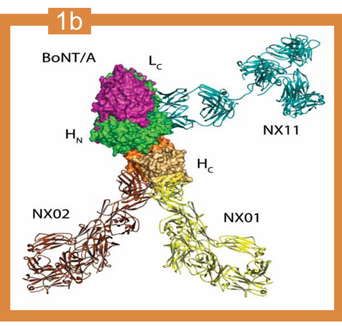
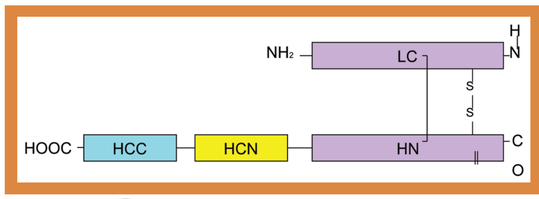


Fig. 1 a. Ribbon cartoon of the X-ray crystal structure of BoNT/A secondary structure (PDB accession name, 3BTA) showing catalytic light chain (LC, magenta), heavy chain (HC, orange) and translocation (HN, green) domains. The belt region of HN, which wraps around the LC, is indicated.

Fig.1 b. Model of the binding of three neutralizing mAbs to BoNT/A. The binding of NX01,NX02 and NX11 IgG to their epitopes on BoNT/A were modeled using the coordinates of BoNT/A (3BTA) and three copies of the coordinates of an arbitrary human IgG (1HZH) using Pymol software (DeLano Scientific, LLC). The model indicates that all three mAbs could bind simultaneously.

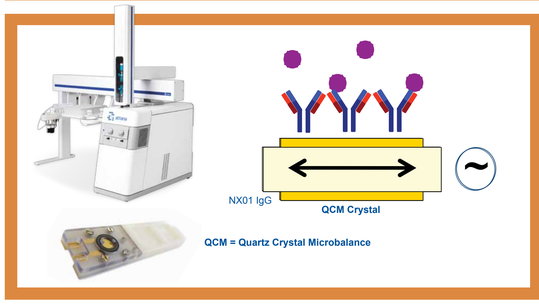
METHOD



HCC expression strategy

Cartoon of the three BoNT/A domains representing the epitopes of NX01, NX02, AND NX11.

In this work, HCC is expressed using pET/BL21 system with a C-terminal 6xhis tag and a SV5 tag. HCC contains amino acids #1092-1296. Mw is 26.9 kDa and the calculated pI=9.25.



HCC assay using Attana sensor technologies

The quartz crystal microbalance technique allows real time, label-free measurement of molecular interaction. It offers fast quantitative measurement of active HCC concentration in different purification samples and can be used for quality control.

In this work, HCC assay consists of the following steps:

1. Monoclonal antibody NX01 is immobilized onto Attana's LNB surface via amine coupling method
2. Crude and purified HCC samples is injected to chip surface
3. Chip surface is regenerated using 2M $MgCl_2$ solution
4. Serial dilutions of highly pure HCC solution was used to construct standard curve for crude sample active concentration determination

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EXPERIMENTAL RESULTS

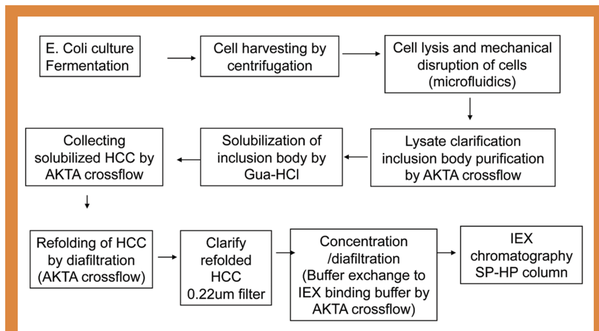

Fig. 2 Purification scheme of BoNT/A domain HCC

1. Cell culture paste (60g wet weight from 10 liter fermentation culture) was re-suspended in lysis buffer and lysed using microfludics.
2. Cell lysate was processed using automated programmed run with AKTA crossflow. Inclusion body was purified, solubilized by 6M Guanidine-HCl, then collected. We use 750kDa cut off hollow fiber cartridg for this process. Each run can handle a total protein of 4000mg.
3. HCC was refolded through diafiltration process using 1xPBS buffer and 10kDa cut off cassette filter, then further concentrated and dialyzed to IEX loading buffer with automated AKTA cross flow run. Each run can handle 1000mg of protein and yield about 100mg of 30% pure total protein
4. SP-HP column is used for ion exchange chromatography purification of HCC. The running buffer is 20mM phosphate, pH7.0. HCC is eluted by NaCl gradient.
5. Final yield of highly pure HCC was about 100mg.

Material	OD	Volume (ml)	Total protein (mg)	Specific activity(active protein/total protein)	Total activity	%Yield	Purification factor
Inclusion body solubilized	1.77	405.00	716.85				
Refolded in 1xPBS	0.33	650.00	214.50	0.32	68.64	100.00	1
Concentrated and dialyzed	0.51	150.00	76.50	0.32	24.48	35.66	1
IEX	1.00	20.00	20.00	1.00	20.00	29.14	3.1

Table 1 Purification table of BoNT/A domain HCC

* Specific activity was calculated based on Attana binding assay's result (see below in Fig. 7 and 8)

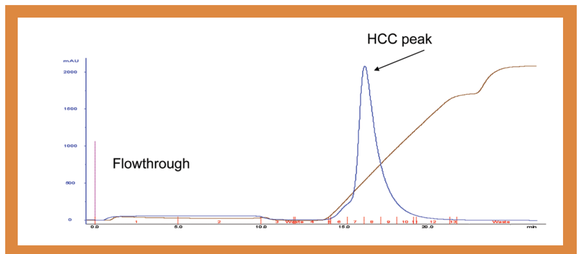

Fig. 3 Chromatogram of HCC purification by ion exchange chromatography. HCC is positively charged (pI: 9.25) in 20mM sodium phosphate buffer, pH7.0. It binds to cation column SP-XL and can be eluted with NaCl salt gradient.

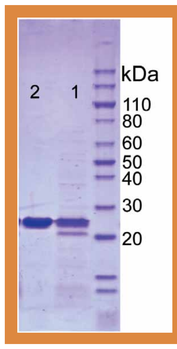

Fig. 4 SDS-PAGE analysis of purified HCC

Lane 1:
HCC after refolding by diafiltration

Lane 2:
HCC after Ion exchange chromatography

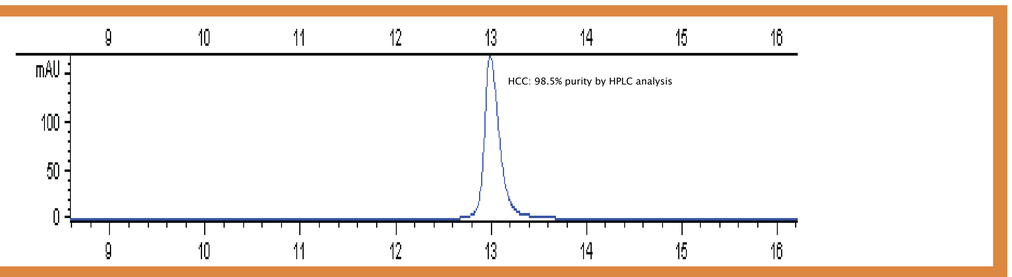

Fig. 5 HPLC analysis on purity of HCC after IEX

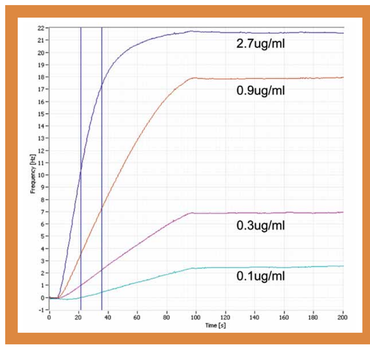

Fig. 6 Binding response curves of HCC to immobilized NX01 on chip surface

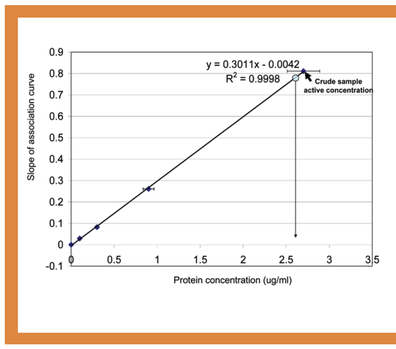

Fig. 7 Standard curve for active concentration determination for HCC

CONCLUSIONS

1. A bacteria expression system that generates hundred milligrams of pure HCC each batch is established.
2. A procedure of automated processing of inclusion bodies and refolding of HCC was optimized using AKTA crossflow system, the purity of HCC is over 98% after Ion exchange chromatography.
3. Attana's quartz crystal microbalance technology is helpful in monitor active protein concentration during the whole HCC purification and production process.