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Evaluation of anti-botulinum neurotoxin single domain antibodies with additional optimization for improved production and stability

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ABSTRACT

Botulinum neurotoxin (BoNT) is a highly potent and lethal toxin, which even in minute quantities can lead to death. BoNT occurs in seven well described serotypes, A-G, and it is critical to not only detect the presence of BoNT, but also to determine the serotype to which a person has been exposed, as the degree of toxicity and persistence of symptoms varies greatly between the various types. Recently, Conway et al. 2010 developed single domain antibodies (sdAb), the recombinant variable domains of heavy-chain-only antibodies derived from camelids, for the detection of all seven serotypes of BoNT; identifying pairs of sdAb for each serotype they demonstrated the sensitive detection of each toxin. Using the sequence information provided in that work, fourteen of their sdAb were recreated with one goal being confirmation of their binding ability and specificity for the seven serotypes of BoNT. This was accomplished using a direct binding assay with the toxins immobilized on microtiter plates. In addition, the melting temperatures and production yields from *E. coli* shake flask fermentation were determined for each of the sdAb produced. In several instances, alternatives or variants of the previously described sdAb were prepared, either to improve the stability or production yields of the anti-BoNT sdAb. Insertion of four framework 1 point mutations (1E or D, 3Q, 5V, and 6E) gave repeated improvement in thermal stability by 5–9 °C, offering a method for increasing sdAb melting temperatures. This work provides for the independent verification of the ability of these sdAb to recognize all seven serotypes of BoNT, furnishing melting temperature, relative affinity, and production yield information that will allow for their future utilization with increased confidence.

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1. Introduction

Botulinum neurotoxins' (BoNTs') status as the “most poisonous poison” (Lamanna, 1959) has warranted their classification by the U.S. government as tier one agents, alongside Ebola virus, Smallpox virus and *Bacillus anthracis*. BoNT is produced by *Clostridium botulinum*, a Gram-positive, rod-shaped, anaerobic, spore-forming, motile bacterium, as a supramolecular complex, wherein the ~150 kDa toxin further associates with neurotoxin accessory proteins (NAPs) to form toxin complexes that range from 300 to 900 kDa in size. The NAPs serve to protect the toxin and thereby greatly enhance its lethality. Once the toxin has been cleaved to its activated form, it is composed of a 100-kDa heavy chain joined via a disulfide bond to a 50-kDa light chain. The heavy chain is responsible for binding specifically to presynaptic nerve terminals, as well as for mediating translocation of the light chain into the cell cytoplasm. The light chain acts as a zinc metalloprotease. After being translocated into the host cell cytoplasm, the light chain cleaves one of the SNARE proteins: SNAP25 (synaptosomal-associated protein), VAMP (vesicle-associated membrane protein), or syntaxin. BoNT A and E cleave SNAP25, BoNT C cleaves both SNAP25 and syntaxin, and BoNT B, D, F, and G target VAMP. This cleavage can have serious implications, for example, the cleaved SNAP-25 is unable to mediate fusion of vesicles with the host cell membrane, thus preventing the release of the neurotransmitter acetylcholine from axon endings, which can result in paralysis and death (Rossetto et al., 2014).

Thus, the ability to rapidly and sensitively detect BoNTs is of vital importance to prevent intoxication. In 2010 Conway et al. described the selection of llama-derived single domain antibodies (sdAb) specific for BoNT serotypes A through G, and identified pairs of capture and tracer sdAb that were incorporated into a multiplexed bead-based sandwich assay for the BoNT serotypes (Conway et al.,

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2010). Using these sdAb pairs, toxins and toxin-complexes were identified in both buffer and assorted food matrices.

SdAb are derived from the variable domain of the heavy-chain-only antibodies found in camelids including camels, llamas and alpacas (Arbabi Ghahroudi et al., 1997; Hamerscasterman et al., 1993). They combine the sensitivity and specificity of conventional antibodies with advantages that come from being comprised of only a single domain, such as the ability to refold after denaturation, excellent solubility, and the capacity to be produced in good yield (de Marco, 2011; Eyer and Hruska, 2012; Muyldermans, 2013). In addition to other applications, sdAb are proving useful as therapeutics, i.e. Caplacizumab (Alzogaray et al., 2011; Harmsen and De Haard, 2007; Peyvandi et al., 2016; Wesolowski et al., 2009).

Since, sdAb are produced recombinantly, most often in *Escherichia coli,* they are amenable to the formation of fusion constructs to tailor their integration into a variety of assay formats and sensor systems (Hussack et al., 2009; Liu et al., 2013, 2016; Pleschberger et al., 2004; Raphael et al., 2015; Sherwood and Hayhurst, 2012). Also they can be altered to improve their biophysical properties; mutagenesis has led to variants with improved protein production and stability, as assessed by the protein's melting point (Anderson et al., 2016; Hagihara et al., 2007; Liu et al., 2015; Saerens et al., 2005, 2008; Turner et al., 2015).

Having verified antibodies available that sensitively and specifically recognize all seven BoNT serotypes is of importance for the development of immunoregents, whether it be for detection, diagnostic, or therapeutic applications. To address this need, we started with the seven sdAb pairs first selected and tested by Conway et al. (2010), reevaluating each for its ability to be produced *in E. coli,* its toxin binding ability and specificity, and finally its melting temperature via a fluorescent dye-based melting assay. Based on our assessment, in several cases we either modified the original clone, or tested an alternative clone previously identified (Conway et al., 2010), and modified versions of the alternative clone. We constructed variants of five sdAbs, a BoNT C binder, two BoNT E binders, a BoNT F binder, and a BoNT G binder. Between the original clones and some alternatives we succeeded in identifying clones that functioned well in the direct binding assay, as well as possessing desirable protein production characteristics and/or an improved melting temperature.

2. Materials and methods

2.1. Cloning

The sequences for the BoNT-binding sdAb A17, A18, B2, B4, C1, C6, C24, D16, D22, E4, E6, E7, F5, F8, F9, G3, and G20 were previously published (Conway et al., 2010). The genes were synthesized (Eurofins Genomics, Louisville, KY) with flanking Nco I and Not I sites to facilitate cloning into pET22b. Similarly, variants of C6, E6. E7, F8 and G3 were synthesized and cloned into pet22b. All cloning enzymes were from New England Biolabs (Ipswich, MA). Sequence alignments were performed using Multalin (Corpet, 1988). The online tool ANARCI (antigen receptor numbering and receptor classification) was used to number the aminio acid sequences of the sdAb (Dunbar and Deane, 2015). In all cases, the IMGT numbering scheme was employed (Lefranc et al., 2003).

2.2. Protein production

Proteins were produced and isolated from the periplasmic space using a protocol similar to the ones described previously (Liu et al., 2015; Turner et al., 2015), with a few modifications. Expression plasmids were transformed into Tuner (DE3) for protein production. Cultures were started from freshly transformed colonies used to inoculate 50 mL of terrific broth (TB) containing 100 μg/mL ampicillin and grown at 25 °C overnight. The next day the overnight culture was poured into 450 mL TB (100 μg/mL ampicillin), grown for 2 h at 25 °C, induced by the addition of 0.5 mM IPTG, and grown an additional 2 h at 25 °C.

Pelleted cells from each 500 mL shake flask culture were resuspended in 14 mL of 100 mM Tris, 0.75M sucrose pH 7.5. Next 1 mL of lysozyme (1 mg/mL) was added followed by 28 mL of 1 mM EDTA added drop-wise to the solution while the centrifuge tubes held in crushed ice were shaking on a rotating platform. After addition of the EDTA, 0.25 mL of 5% deoxycholate was added and the cells were gently swirled for another half hour. Lastly, we added 1 mL of 0.5M $MgCl_2$, incubated for a further 15 min, and pelleted the spheroplasts.

Protein was purified by immobilized metal affinity chromatography followed by size exclusion chromatography as described previously (Liu et al., 2015). Yield was determined by UV spectroscopy using a nanodrop (Thermo Fisher, Waltham, MA). Sodium azide was added to 0.1% and sdAb were stored at 4 °C prior to evaluation.

2.3. Fluorescent dye-based melting assay

The Fluorescent dye-based melting assay (dye melt) was performed as described previously (Turner et al., 2015b; Walper et al., 2014). Each sdAb was diluted to a concentration of 500 μg/mL in a final volume of 20 μL PBS. Next, Sypro Orange dye was added to each sample at a dilution of 1:1000. Finally, samples were measured in triplicate using a StepOne Real-Time PCR machine (Applied Biosystems, Foster City, CA). The heating program was run in continuous mode from 25 °C–99 °C at a heating rate of 1% (~2 °C per minute), and data was recorded using the ROX filter. The melting point was determined to be the peak of the first derivative of the fluorescence intensity. Each measurement was performed in triplicate with all three replicates giving essentially identical values for the melting temperature.

2.4. ELISA

To test the specific activity, relative affinity, and cross-reactivity of the sdAb, ELISAs were performed using commercially available micro-titer plates pre-coated with the various Clostridium botulinum toxins or toxin-complex from METAbiologics, Inc., Madison, WI. For cross-reactivity studies, 100 and 20 ng/mL of each sdAb was tested on plates coated with BoNT A, B, C, D, E, and F toxins and BoNT C, D, and F toxin-complexes. Dose response curves were generated for the sdAb specific for the coated toxin/complex and those that showed high cross-reactivity at 100 ng/mL.

Each of the sdAb was biotinylated as described previously (Goldman et al., 2008; Graef et al., 2011). Next 100 μL of the biotinylated BoNT-binding sdAb was added to wells such that the concentration was 100 ng/mL or lower. Also included were controls including an irrelevant sdAb and blanks. The solution was allowed to incubate for 30 min with shaking. Plates were washed 3 times with PBS buffer using a Biotech plate washer (Winooski, VT). Next, 100 μL of a 1 μg/mL streptavidin-HRP was added, incubated for 30 min with shaking, then the plate was washed 3 times with PBS. To generate the colorimetric readout for the assay 100 μL/well of KPL Sure-Blue (SereCare, Gaithersburg, MD) was added. Once the blue color developed, 100 μL 1N HCl was added to stop color development and the plate read at 450 nm with Tecan Plate Reader (Morrisville, NC). The ELISA data was then analyzed through SigmaPlot 12 using the nonlinear regression ligand binding 1 site saturation plus nonspecific to calculate the relative affinity.