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Expression, purification and characterization of the receptor-binding domain of botulinum neurotoxin serotype B as a vaccine candidate

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ABSTRACT

The receptor-binding domain of botulinum neurotoxins (the H_C fragment) is a promising vaccine candidate. Among the H_C fragments of the seven BoNT serotypes, the expression of H_C/B in *Escherichia coli* is considered especially challenging due to its accumulation as a non-soluble protein aggregate. In this study, the effects of different parameters on the expression of soluble H_C/B were evaluated using a screening assay that included growing the bacterium at a small scale, a chemical cell lysis step, and a specific ELISA. The highest soluble H_C/B expression levels were obtained when the bacterium *E. coli* BL21(DE3) + pET-9a-H_C/B was grown in terrific broth media at 18 °C without induction. Under these conditions, the yield was an order of magnitude higher than previously reported. Standard purification of the protein using a nickel column resulted in a low purity of H_C/B. However, the addition of an acidic wash step prior to protein elution released a major protein contaminant and significantly increased the purity level. Mass spectrometry analysis identified the contaminant as ArnA, an *E. coli* protein that often contaminates recombinant His-tagged protein preparations. The purified H_C/B was highly immunogenic, protecting mice from a 10^6 LD_{50} challenge after a single vaccination and generating a neutralizing titer of 50 IU/ml after three immunizations. Moreover, the functionality of the protein was preserved, as it inhibited BoNT/B intoxication *in vivo*, presumably due to blockade of the neurotoxin protein receptor synaptotagmin.

Introduction

Botulism is a neurologic disease caused by toxins mainly produced by the bacterium *Clostridium botulinum*. Although botulism cases are rare in humans, botulinum neurotoxins (BoNTs[1]) are the most poisonous substances known, and they are therefore categorized as a tier 1 select agent by the Center for Disease Control and prevention (CDC) [1]. There are seven serologically distinct serotypes of these neurotoxins (designated A–G), among which serotypes A, B, and E are the most abundant in cases of human intoxication [2]. BoNTs are 150-kDa proteins, consisting of a 100-kDa heavy chain (H) joined to a 50-kDa light chain (L) *via* a disulfide bond. The toxins of all serotypes share a similar architecture, organized into three structural domains that mediate the three steps of the intoxication process. The first step is the attachment of the receptor-binding domain, located at the C-terminus of the heavy chain (the H_C fragment), to its receptors and subsequent internalization via endocytosis. The next step is the translocation and release of the light chain into the cytosol, a step considered to be facilitated by the translocation domain found on the N-terminus of the heavy chain (H_N). The final step is the cleavage of one of three SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptor) proteins by the light chain, which possesses endopeptidase activity, thereby inhibiting the release of the neurotransmitter acetylcholine from nerve cells to synapses [2–4].

The prevention and treatment of botulism is based on the presence of toxin-specific neutralizing antibodies in the blood stream. The source of the antibodies can be either self, in the case of prophylactic vaccination, or external (usually of vaccinated equine origin), in cases of post-exposure treatment. Historically, vaccines against botulism have consisted of formalin-inactivated toxins (toxoid) adsorbed to aluminum hydroxide [3]. Although toxoid-based botulinum vaccines are safe and efficient [3,5], their manufacturing process is expensive because it requires large-scale production facilities for this highly hazardous and spore forming agent. Therefore, current efforts to develop botulism vaccines are mainly focused on subunit vaccines consisting of the H_C fragment.

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[1] *Abbreviation used:* BoNTs, botulinum neurotoxins; CDC, Center for Disease Control and prevention; rBV, recombinant botulinum vaccine; TB, terrific broth; CV, column volumes; TTD, time to death.

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The H_C fragment is not toxic by itself, but it contains many neutralizing epitopes and can be produced recombinantly. In 1995, Clayton et al. reported that vaccination of mice with a recombinant H_C fragment of neurotoxin serotype A elicited protective immunity against challenge with its homologous toxin [6]. Since that time, extensive research efforts have been made, and the receptor-binding domains of the seven BoNT serotypes have been produced and shown to induce a protective immune response [5,7]. Moreover, a recombinant botulinum vaccine (rBV) composed of the H_C fragments of botulinum neurotoxins A and B, produced by the DynPort Vaccine Company for the United States Department of Defense, is currently under clinical investigation [8].

The first attempts to produce recombinant H_C fragments of neurotoxin A were performed using *Escherichia coli* as a host [6,9]. However, as most of the expressed protein was insoluble in this system, subsequent studies have used the alternative host *Pichia pastoris*. Using this system, the H_C fragments of botulinum A, B, C, D, E, and F have been produced with satisfactory yields [10–15]. Recently, the interest in expressing H_C fragments in *E. coli* has returned, with better success. In the expression of the receptor domains of BoNT/E and BoNT/F, yields of 8–20 mg of protein per liter of culture were obtained [7,16,17]. Greater efforts have been made regarding H_C/A expression. Several groups reported yields of 10–70 mg of H_C/A per liter of culture [7,18,19], and we recently developed an expression system that yields 350 mg of H_C/A per liter [20]. Nevertheless, expression of the receptor-binding domain of BoNT/B has remained challenging, as the reported yields of the expression of this domain are much lower. In the work of Baldwin et al. [7], the H_C fragments of seven BoNT serotypes were expressed in *E. coli* to produce a heptavalent botulinum vaccine. The authors described special difficulty in expressing H_C/B, as most of the protein was found in the insoluble cell fraction, with only ~2 mg of the protein being obtained from 1 liter of culture. Similar yields were reported by Held et al. (1 mg per liter) [21]. Gao et al. purified H_C/B from inclusion bodies with higher yields [22]. However, proper refolding of the protein into its native structure could not be achieved, and the final protein was obtained in 2 M urea.

In this study, we developed a simple, efficient, and economic process for expressing H_C/B in a soluble form in *E. coli*, achieving a yield that is an order of magnitude higher than previously reported. The protective properties of the recombinant product are at least comparable to those of H_C/Bs produced using other expression systems, and the product is functionally active, as it was able to inhibit BoNT/B intoxication *in vivo*. During the development of the purification process, we found that a simple acidic wash step is able to remove ArnA, an *E. coli* protein that frequently contaminates His-tagged protein preparations due to its affinity for nickel ions. This finding has broad implications for the purification of other recombinant proteins.

Materials and methods

Ethics statement

All animal experiments were performed in accordance with the Israeli law and were approved by the Ethics Committee for Animal Experiments at the Israel Institute for Biological Research.

Materials

All chemicals were purchased from Sigma–Aldrich or Merck, unless otherwise stated. Yeast extract and tryptone were obtained from Becton, Dickinson and Company (Franklin Lakes, NJ). Mouse anti-H_C/B monoclonal antibody was prepared as previously described [23]. Rabbit anti-H_C/B polyclonal antibodies were purified from the sera of hyperimmune rabbits that were immunized with H_C/B, as previously described [24]. A synthetic H_C/B gene with optimized codon usage for expression in *E. coli* was synthesized by GenScript (Piscataway, NJ).

Bacteria and toxins

E. coli BL21(DE3) and the pET-9a plasmid were purchased from Novagen (Madison, WI). *Clostridium botulinum* strain B was obtained from the IIBR collection (B592). The neurotoxin gene of this strain complies with that of the Danish strain (Accession Number M81186) [25]. BoNT/B was prepared from concentrated supernatant of a culture grown for 6 days in anaerobic culture tubes.

Growth of cultures for optimization experiments

During optimization, the cells were grown with shaking (250 rpm) in 250-ml polycarbonate baffled shake flasks (Nalgene; Rochester, NY) containing 40 ml of media and kanamycin (30 μg/ml). The cultures were inoculated with a 1:100 dilution of a starter culture grown in terrific broth (TB) media. The following media were evaluated: TB (12 g/l tryptone, 24 g/l yeast extract,0.4% (v/v) glycerol, and 89 mM potassium phosphate); TB + sorbitol (660 mM sorbitol, 12 g/l tryptone, 24 g/l yeast extract, 0.4% (v/v) glycerol, 89 mM potassium phosphate, and 2.5 mM betaine); and minimal media [26] (30 g/l glycerol, 13.3 g/l KH_2PO_4, 4 g/l $(NH_4)_2HPO_4$,1.7 g/l citric acid, 1.2 g/l $MgSO_4{\cdot}7H_2O$, 8.4 mg/l EDTA, 2.5 mg/l $CoCl_2{\cdot}6H_2O$, 15 mg/l $MnCl_2{\cdot}4H_2O$, 1.5 mg/l $CuCl_2{\cdot}2H_2O$, 3 mg/l H_3BO_3, 2.5 mg/l $Na_2MoO_4{\cdot}2H_2O$, 13 mg/l $Zn(CH_3COO)_2{\cdot}2H_2O$, 100 mg/l Fe(III)citrate, and 4.5 mg/l thiamine·HCl.

Soluble H_C/B quantification assay

This assay was used to estimate the yield of the soluble H_C fragment in cultures grown under various conditions during expression optimization. The assay included two steps. First, samples (0.5 ml) withdrawn from the cultures were chemically disrupted with the CelLytic B Plus Kit (Sigma–Aldrich) according to the manufacturer's instructions, and the soluble proteins were separated from the insoluble cell fraction by centrifugation. The concentration of the H_C fragment in the supernatants was then estimated by sandwich ELISA as follows. Plates (Maxisorp, Nunc; Roskilde, Denmark) were coated with 50 μl of a mouse anti-H_C/B monoclonal antibody [23] diluted to a final concentration of 4 μg/ml in coating buffer (50 mM Na_2CO_3, pH 9.6) and then incubated overnight at 4 °C. The plates were subsequently washed with wash solution (0.9% NaCl, 0.05% Tween 20) and blocked for 1 h at 37 °C with TSTA buffer (50 mM Tris, 0.9% NaCl, 0.05% Tween 20, 2% BSA, 200 μl per well). After washing, the plates were incubated with serial dilutions (50 μl per well, in duplicate) of the tested supernatant and pure H_C standard in TSTA for 1 h at 37 °C. The plates were then washed with wash solution and incubated for 1 h with a rabbit anti-H_C fragment polyclonal antibody, diluted in TSTA to a final concentration of 0.5 μg/ml. After additional washing, the plates were incubated with 50 μl of alkaline phosphatase-conjugated donkey anti-Rabbit IgG (Jackson ImmunoResearch) diluted 1:1,500 for 1 h at 37 °C. Finally, the plates were washed with wash solution, and the colorimetric reaction was developed using the substrate *p*-nitrophenyl phosphate (1 mg/ml in 0.2 M Tris buffer). The absorbance at 405 nm was continuously measured for 15 min in 30-s intervals, and the concentration of the H_C fragment was determined by interpolation from a standard curve of H_C/B.