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Research Article

Recombinant synthesis and immunological characterization of single-domain antibodies against botulinum neurotoxin serotype A and B

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Abstract

Nanobodies which are single-domain antibodies derived from the Variable Heavy domain of Heavy-chain-only antibodies (VHHs) found in camels and sharks, represent a novel approach for enhancing the detection and neutralization of food toxins. Botulinum neurotoxin (BoNT), a potent neurotoxin produced by *Clostridium botulinum*, can cause severe poisoning even in minimal amounts (approximately 1 - 2 ng/kg), necessitating the development of highly sensitive detection methods. In this study, we recombinantly synthesized the nanobodies ALc-B8 (A8) and JNE-B10 (J10), which specifically bind to the light chains of BoNT type A (BoNT/A) and BoNT type B (BoNT/B), respectively. Optimal conditions for expressing nanobodies A8 (33 kDa) and J10 (32 kDa) were identified using the pET28a vector in *Escherichia coli* BL21 (DE3) RIL bacteria, with induction by 0.5 mM IPTG at an OD_{600} of approximately 0.6 - 0.8 for 16 - 18 hours at 16°C. The purified nanobodies A8 and J10, yielding 10 - 20 mg/L of culture, were able to specifically recognize the recombinant BoNT/A and BoNT/B light chains at a concentration of 100 µg/mL in Western blotting. These findings provide a foundation for the application of these nanobodies in the detection and neutralization of botulinum toxins.

Keywords: *Nanobody, VHH, botulinum, food toxin, neutralization, recombinant protein*

1. INTRODUCTION

Nanobodies, also known as single-domain antibodies, are the recombinant variable heavy domain of heavy chain-only antibodies. Heavy chain-only antibodies have been naturally found in camels and sharks, where they are produced as part of the immune system's response to foreign antigens. Despite lacking a light chain, nanobodies exhibit several advantages over conventional antibodies including high thermal stability, good solubility, and low immunogenicity. With a molecular weight of approximately 13 - 15 kDa, nanobodies can be easily synthesis using DNA recombinant technology once the genetic sequences are identified [1]. Additionally, their production with known sequences is independent of antigen purification, which is advantageous when working with antigens that are difficult to isolate or unstable during purification.

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Botulinum neurotoxin (BoNT) is among the most dangerous neurotoxins with an average lethal dose (LD50) in humans ranging from approximately 1.3 to 2.1 ng/kg via intravenous/intramuscular (IV/IM) administration and from 10 to 13 ng/kg via inhalation route [2]. BoNT consists of seven serotypes (BoNT/A-G), among which BoNT/A and BoNT/B pose the highest risk to humans due to their high toxicity and prolonged duration of action [3]. Epidemiological data from the United States (1980-1996) indicate that, among 135 cases of foodborne botulism, the porpotions caused by type A, B, E, F, and unspecified types were 54.1%; 14.8%; 26.7%; 1.5%; and 3.0%, respectively [4]. In humans, BoNT/A, B, and E are the primarily attributed poisoning toxins. Upon entering the neuron, BoNT binds specifically via its heavy chain to its neuronal receptor, which facilitates the internalization and translocation of the light chain (LC) into the cytoplasm. LC then acts as a protease that cleaves SNARE protein complex in the neuron, thereby inhibiting the release of acetylcholine and disrupting neuronal signaling [5].

Currently, a promising approach using next-generation antibodies, which are nanobodies, has been developed to enhance both the recognition and inhibition of BoNT activity [6, 7]. Based on previous studies, two nanobodies, ALc-B8 (A8) and JNE-B10 (J10), were selected for recombinant expression. These two nanobodies have demonstrated high-affinity and specificity binding to LC of BoNT/A and BoNT/B [5, 6, 8, 9]. The ability to synthesize nanobodies with known sequences secure an efficient domestic supply of recombinant antibodies, supporting their application in both toxin detection and therapeutic neutralization.

2. MATERIALS AND METHOD

2.1. Materials

E.coli BL21 (DE3) RIL, Novagen (USA); pET28a(+) vector containing codon-optimized sequence for A8 (pET28a-A8) (GenBank: ACS73863.1) [9] and J10 (pET28a-J10) (PDB: 7T5F_B) [5] were synthesized by GenScript Biotech (USA). Recombinant BoNT/A antigen was provided by Joint Vietnam-Russia Tropical Science and Technology Research center.

2.2. Chemicals and standards

GangNam-STAIN™ Prestained Protein Ladder, iNtRON (South-Korea); GeneRuler 100 bp and 1 kb DNA Ladder (Clever, UK); restriction enzyme BamHI và HindIII (NEB, USA); HisPurTM Ni-NTA Resin purification column (Thermo, USA); DNA gel extraction kit and plasmid extraction kit (NEB, USA); Rabbit anti Sheep IgG (H+L) Secondary antibody, AP (ThermoFisher), Goat anti-Llama IgG H&L (HRP) (Abcam).

2.3. Research methodology

2.3.1. Transformation of pET28a-A8 and pET28a-J10 plasmids into E. coli bacteria

BL21 (DE3) RIL cell was thawed on ice from -80°C for about 10 - 20 minutes. Next, 120 ng of pET28a-A8/J10 plasmid was gently mixed with the competent cells and incubated on ice for 20 minutes. The mixture was heat-shocked at 42°C for 60 seconds, then transferred to ice for 5 minutes. Subsequently, 500 - 700 µL of antibiotic-free LB medium was added to the mixture. The mixture was then incubated at 37°C with shaking at 150 rpm for 45 - 50 minutes. Cells were collected by centrifugation at 2300 rpm for 10 minutes and then plated on a solid LB agar supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. The plates were cultured overnight at 37°C.

2.3.2. Determination of the presence of commercial plasmids by PCR

The plasmids were transformed into *E. coli* strains. To confirm the success, random colonies were selected for colony PCR. Both colonies and plasmids isolated from colonies were amplified using the vector primer pair Fw-T7 (5' TAATACGACTCACTATAGGG 3') and Rv-T7 (5' GCTAGTTATTGCTCAGCGG 3'). The expected amplicon sizes were 1020 bp for pET28a-A8 and 1073 bp for pET28a-J10.

2.3.3. Expression and acquisition of total protein extract containing A8/J10 [6]

The screened *E.coli* (DE3) colonies containing plasmids were inoculated in a liquid LB medium supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol and cultured at 37°C. Once the optical density at 600 nm (OD_{600}) of the culture reached 0.5-0.8, Isopropyl (-D-1-thiogalactopyranoside (IPTG) was added at varying concentrations from 0 nM to 1 mM. The cultures were continued to be cultured at 16°C for 18 hours. Cell biomass was collected by centrifugation at 10000 rpm for 10 minutes and stored at -20°C.

The biomass was resuspended in lysis buffer containing 20 mM tris-HCl (pH 7.5), 500 mM NaCl, 10% glycerol, 20 mM imidazole, 1 mM phenylmethylsulfonyl fluoride (PMSF) and then sonicated on ice (4 cycles of 30 sec each). The lysate was then centrifuged at 10.000 rpm for 10 minutes at 4°C to collect the supernatant as the total protein extract. The expression levels of the A8 and J10 nanobodies were analyzed by SDS- PAGE electrophoresis and Western blotting.

2.3.4. Purification of A8 and J10 nanobodies

The protein extract was loaded onto the Ni-NTA affinity chromatography column, the flow-through (Ft) was collected, and the column was then washed with a wash buffer until the recorded absorbance at 280 nm (A280) was below 0.05. Next, the elution buffer containing 250 mM imidazole was used to elute the bound protein from the column. The purified A8 and J10 nanobodies were passed through the PD10 desalting column (Cytiva Sephadex G-25) to remove residual imidazole. Finally, the concentration of protein was determined by measuring absorbance at 280 nm.

2.3.5. SDS-PAGE Protein Electrophoresis [10]

Protein electrophoresis was conducted using SDS-PAGE following Laemmli's method [10]. Samples were prepared by mixing the protein solution with sample buffer containing 032 mM Tris base, 50% glycerol, 17 mM SDS, 2% mercaptoethanol, bromophenol blue and then heat treated at 95°C for 5 minutes and subsequently cooled on ice. After electrophoresis, the gel was stained with a 2.5% CBB solution (w/v) and destained with a methanol-acetic acid solution until a clear background was observed.

2.3.6. Dot blotting

Antigens were spotted on the nitrocellulose membrane. Next, the membrane was incubated with nanobody A8 or J10 as the primary antibody at varying concentrations of 0, 50, 100, and 200 µg/mL. The secondary antibody HRP-binding Anti-Llama (Abcam, Cat. ab112786) was used to detect the affinity binding.

2.3.7. Western blotting

Western blotting were performed for two main purposes: confirmation of the presence of A8, J10 and assessment of their immunological activity. For detection, A8 and J10 nanobodies were separated on SDS-PAGE gel and transferred to nitrocellulose membranes, and probed with the primary anti-His-tag monoclonal antibodies (mouse anti-histidine),followed by an AP-conjugated anti-mouse secondary antibody.

For the evaluation of the immunoreactivity, LC-BoNT/A, B antigens were electrophoresed and transferred to the nitrocellulose membrane, which was then incubated with A8 /J10 as the primary antibody. The second antibody used was HRP-conjugated Anti-Llama (Abcam, Cat. ab112786). Commercial antibodies used as controls included: C. botulinum BoNT-A Light Chain Polyclonal Antibody (sheep) (R&D Systems, Cat. PA5-48053) and C. botulinum BoNT-B Light Chain Polyclonal Antibody (sheep) (Invitrogen, Cat. PA5-47737), with AP-conjugated anti-sheep secondary antibody (Invitrogen, Cat. 31360).

2.3.8. ELISA

LC-BoNT/A, B antigens were coated on the 96-wells plate overnight. The coated plates were incubated with A8 or J10 as the primary antibodies. Afterward, The seconde gary antibody used was HRP-conjugated Anti-Llama (Abcam, Cat. ab112786).

3. RESULTS AND DISCUSSION

3.1. Evaluation of nanobody expression

The pET28a-A8 and pET28a-J10 plasmids were successfully transformed into *E. coli* BL21 (DE3) RIL bacteria, as confirmed by PCR amplification with vector-specific primers. The electrophoretic analysis of PCR showed the bands of the expected size: 1020 bp for pET28a-A8 and 1073 bp for pET28a-J10 (**Figure 1A, B**). These results confirmed that the target plasmids were correctly introduced into the bacterial host.

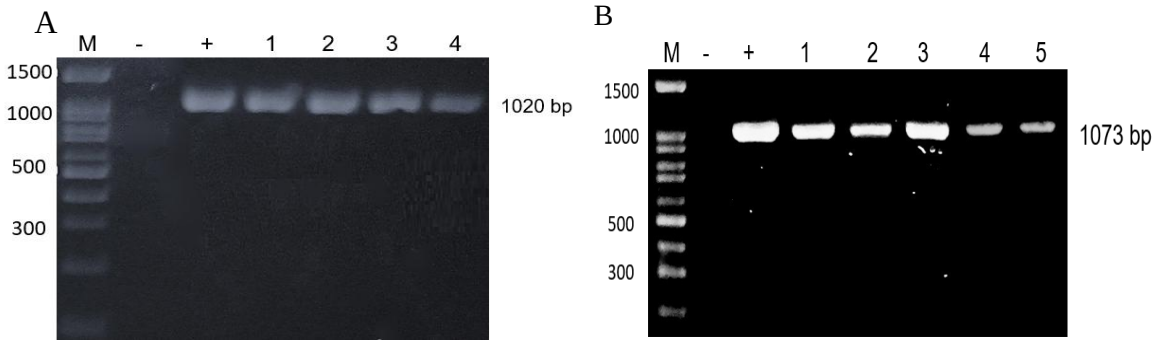

Figure 1. *Confirmation of plasmid transformation into BL21 (DE3) RIL using PCR with Fw-T7 promoter and Rv-T7 terminator primers; Agarose gel electrophoresis of PCR products from colonies 1–4 of nanobody A8 (A) and colonies 1–5 of nanobody J10 (B). M: 100 bp DNA ladder; (−): negative control (H_2O); (+): synthesized plasmid prior to transformation*

Protein expression was optimized by inducing various IPTG concentrations at 16°C with different IPTG concentrations (0 mM to 1 mM). The SDS- PAGE analysis revealed strongly overexpression bands at approximately 33 kDa and 32 kDa - corresponding to nanobodies A8 and J10, respectively. These bands were most prominent at an IPTG concentration of 0.5 mM (**Figure 2**), which aligns with previous reports, such as the expression at 0.4 mM IPTG in the Miyashita study [6]. Additionally, a comparison of the expression of nanobodies in the supernatant and pellet fraction reveals the difference in solubility of A8 and J10 nanobodies. While the majority of nanobody A8 was distributed in the soluble fraction, the nanobody J10 was equivalently present in both the soluble and insoluble (pellet) fractions. However, the soluble amount of J10 in the supernatant fraction was sufficient for use in subsequent experiments.

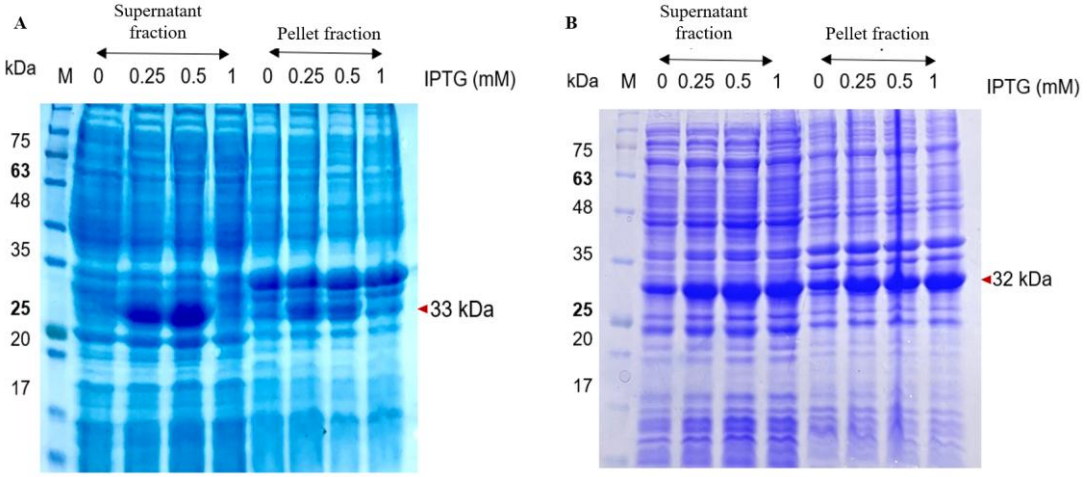

Figure 2. *Expression of A8 and J10 nanobodies at different IPTG concentrations. SDS-PAGE analysis of nanobody A8 (A) and J10 (B) expression. Red arrows indicate the bands corresponding to the expected molecular weights of the nanobodies. M: Protein ladder*

3.2. Purification of A8 and J10 nanobodies

His-tagged A8 and J10 nanobodies in *E. coli* total protein extracts were purified by Ni-NTA affinity columns. The purification results demonstrated efficient purification, yielding approximately 10 - 20 mg of purified protein per liter of culture. The purification of nanobodies was further evaluated by Western blotting utilizing an anti-histidine antibody to detect the His-tags on A8/J10. The observed protein bands corresponded to the expected molecular weights of approximately 33 kDa for A8 and 32 kDa for J10, confirming the identity of the purified proteins as nanobodies A8 and J10 (**Figure 3**).

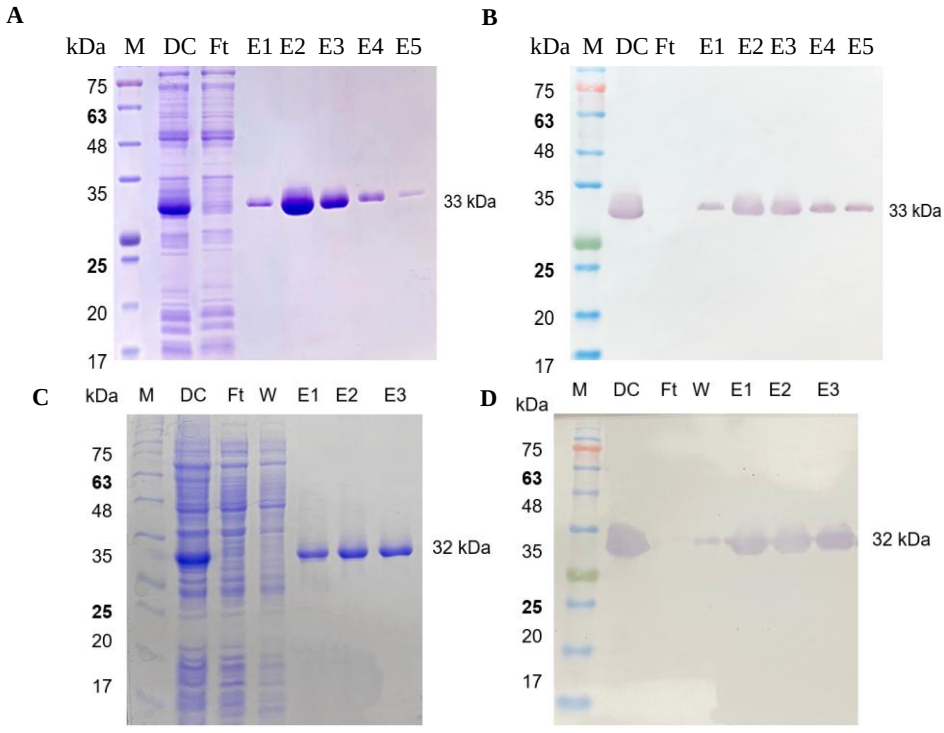

***Figure 3**. A8 and J10 purification results*

*The A8 nanobody purification results were shown on the SDS-PAGE (A) and evaluated by Western blotting (B**).** The results of J10 nanobody purification are shown on SDS-PAGE (C) and confirmed by Western blotting (D). M: Protein ladder; DC: crude protein extract; Ft: flow-through fraction; W: wash fraction; E1, E2, E3, E4, E5: eluted fractions released using 250 mM imidazole*

3.3. Evaluation of immunoreactivity of A8 and J10 Nanobodies with BoNT/A and BoNT/B

The purified A8 and J10 nanobodies were further desalted by PD-10 columns and examined the immunological activity with BoNT/A and BoNT/B recombinant antigens. In order to determine the working concentration of nanobodies, dot blotting was conducted. Nanobody A8 was tested at concentrations of 0 µg/mL, 50 µg/mL, 100 µg/mL, and 200 µg/mL against 0.3 µg of recombinant BoNT/A antigen.

The results indicated that 100 µg/mL was the optimal concentration for detecting recombinant BoNT/A using nanobody A8 (**Figure 4)**. Based on this, 100 µg/mL was also selected for further assays for both A8 and J10. This working concentration is approximately 200–500 times lower than that typically required for commercial antibodies.It is anticipated that antigen recognition by the nanobodies could be further enhanced upon removal of the purification tags.

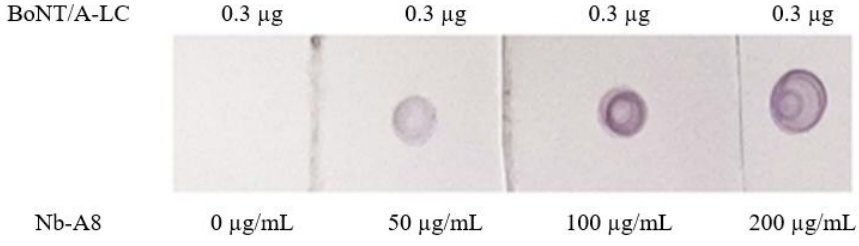

***Figure 4.** Determination of the nanobody working concentration of nanobody A8. LC-BoNT concentration of 0.3 µg was spotted on a nitrocellulose membrane. The membrane was then incubated with nanobody A8 at various concentrations*

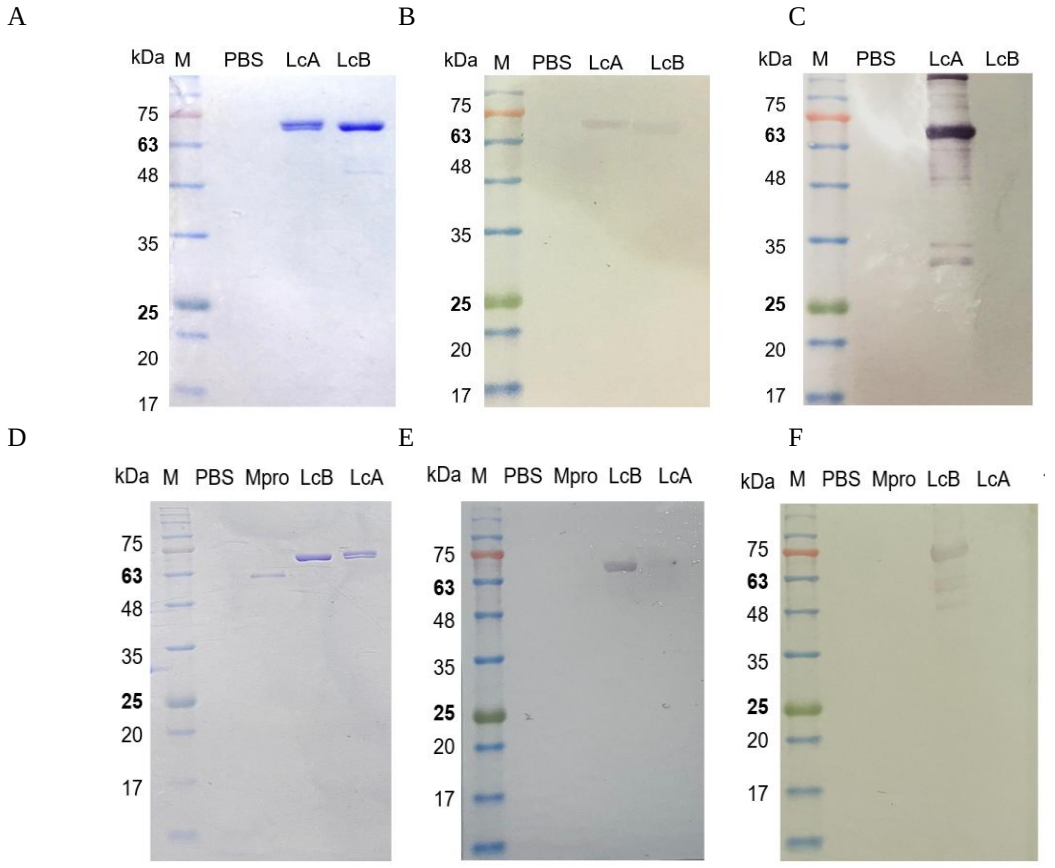

Figure 5. *Evaluation of the antigen-binding specificity of nanobodies A8 and J10 by Western blotting. SDS-PAGE of recombinant LC-BoNT/A and LC-BoNT/B antigens (A, D). Binding specificity to LC-BoNT/A of nanobody A8 (B) and of a commercial antibody (C). Binding specificity to LC-BoNT/B of nanobody J10 (E) and of a commercial antibody (F).M: Protein ladder; PBS, Mpro: negative controls; LcA: LC-BoNT/A; LcB: LC-BoNT/B*

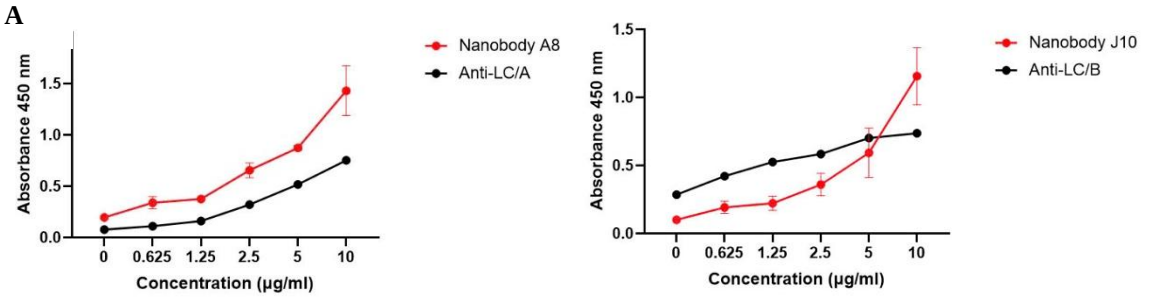

Figure 6. *Evaluation of the antigen-binding specificity of nanobodies A8 and J10 by ELISA. LC-BoNT/A and LC-BoNT/B antigens at varying concentrations were tested using nanobody A8 (red curve) and a respective commercial antibody anti-LC/A (black curve) (A), or nanobody J10 (red curve) and a respective commercial antibody anti-LC/B (black curve) (B). HRP-conjugated anti-llama IgG was used as the secondary antibody*

Western blotting results showed that both nanobodies A8 and J10 recognized BoNT/A and BoNT/B recombinant antigens, as indicated by the appearance of specific bands corresponding to the expected 70 kDa recombinant BoNT antigens (**Figure 5**). Notably, nanobody A8, in addition to specifically detecting LC-BoNT/A, also exhibited cross-reactivity with LC-BoNT/B at approximately 20% (**Figure 5B**), but no cross-binding was observed for the nanobody J10 (**Figure 5E**). ELISA results further confirmed the antigen recognition ability of A8 toward BoNT/A (**Figure 6A**) and J10 toward LC-BoNT/B (**Figure 6B**). The ELISA

signal increased as the concentration of the antigen increased, with the strongest signal observed at 10 µg/mL antigen when using 10 µg/mL of nanobody.

Previous studies have employed nanobodies A8 and J10 for toxin neutralization and antigen-binding structural analyses [6, 8]. However, these studies did not explicitly report expression results or direct analyses of antigen-binding specificity. Furthermore, most of the previous research has focused on characterizing the specific binding properties of the nanobodies in their native form, after removing the fusion tags such as TrxA and His-tags. And to date, no publications have demonstrated the antigen-binding activity of these two nanobodies in their fusion forms. In contrast, this study clearly demonstrated that the fusion forms of A8 and J10 nanobodies remain functional and capable of antigen recognition. This offers advantages in downstream experiments by eliminating the need for tag removal and additional purification steps. Nonetheless, further comparative studies between the native and fusion-tagged forms are required to confirm which platform is the optimal performance.

The generation of these single-domain nanobodies with high antigen specificity opens a wide range of applications. For example, prior studies have shown that nanobody A8 can neutralize BoNT/A activity by binding to the enzymatic active site of the BoNT/A light chain [6, 8], highlighting its potential for both toxin neutralization and toxin detection [6]. In contrast, J10 binds to a non-catalytic epitope, making it suitable for toxin detection applications.

For detection purposes, both A8 and J10 antibodies can be used in the development of biosensors or lateral flow assays (LFA). Additionally, these nanobodies could be immobilized on the magnetic particles to pre-concentrate and isolate BoNT from the complex food matrices, thereby enhancing the detection sensitivity. Several studies have reported that the small size of nanobodies leads to challenges in conjugating on material surfaces, such as ELISA plates, magnetic particles, biosensors, etc. [11]. Therefore, increasing the size of nanobodies is advantageous for improving the immobilization efficiency of nanobodies on the material surface. Our study confirms that the activity of nanobodies was maintained in fusion forms (e.g., with TrxA) suggesting the feasibility of using such fusion constructs for material surface immobilization in BoNT antigen detection.

4. CONCLUSION

Nanobodies A8 and J10 were successfully expressed on *E. coli* BL21 (DE3) RIL under induction with 0.5 mM IPTG for 16 -18 hours at 16°C. Both nanobodies were purified with high purity using Ni-Sepharose, yielding approximately 10-20 mg per liter of culture medium. The optimal concentration of the A8 and J10 nanobodies in dot blotting and Western blotting was determined to be 100 µg/mL. The strongest ELISA signal for recognizing the respective antigens (at 10 µg/mL of antigen concentration) was observed when using 10 µg/mL of nanobody.

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