Vietnam Journal of Food Control

A Scientific Journal of National Institute for Food Control
Journal homepage: https://vjfc.nifc.gov.vn

Research Article

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

Luong Trung Hieu[1*], Bui Thi Thu Hoai[1*], Phan Thi Thanh Thuy[1], Nguyen Le Duc Hai[1], Nguyen Thi Anh Nguyet[1], Pham Bao Yen[1,2], Nguyen Thi Hong Loan[1,2], Phan Tuan Nghia[1,2], Nguyen Thanh Trung[3], Le Phuong Linh[1,4], Trinh Van Toan[5], Le Thi Hong Nhung[3†]

[1]Faculty of Biology, University of Science, Vietnam National University, Hanoi, Vietnam
[2]Key Laboratory of Enzyme and Protein Technology, University of Science, Vietnam National University, Hanoi, Vietnam
[3]National Institute for Food Control, Hanoi, Vietnam
[4]VinUni-Illinois Smart Health Center, Hanoi, Vietnam
[5]Institute of Tropical Medicine, Joint Vietnam-Russia Tropical Science and Technology Research Center, Hanoi, Vietnam

(Received: 25 Oct 2024; Revesed: 05 Dec 2024; Accepted: 05 Dec 2024)

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

Nanobody referred to as single domain antibody, is the recombinant synthesized variable heavy domain of heavy chain (VHH) of antibody. The presence of nanobody was naturally found in camels and sharks, where they are produced as part of the immune system's response to recognize the antigens. Despite the lack of a light chain, nanobodies exhibit several advantages compared with conventional antibodies such as high thermal stability, high solubility, and low immunogenicity. With a molecular weight of approximately 13 - 15 kDa, nanobody can be simply recombined in *in vitro* once the sequence has been identified [1]. The recombination of nanobodies with known sequences is independent of the separation of antigens which also is an advantage because of the low recovery of a few proteins or antigens after imputity.

[*]*Authors with equal contribution*
†Corresponding author: Le Thi Hong Nhung (E-mail: nhungle@hus.edu.vn)
https://doi.org/10.47866/2615-9252/vjfc.4454

Botulinum neurotoxin (BoNT) belongs to the top of the most dangerous neurotoxin with an average lethal dose (LD50) in humans ranging from approximately 1.3 to 2.1 ng/kg via IV/IM administration and 10 to 13 ng/kg via inhalation route [2]. BoNT consists of seven serotypes (BoNT/A-G), among which BoNT/A and BoNT/B present the highest risk in humans because of their high toxin and prolonged duration of action [3]. Epidemiological data from the United States from 1980 to 1996 indicate that among 135 cases of food poisoning the number of cases caused by type A, type B, type E, type F, and unspecified type was 54.1%; 14.8%; 26.7%; 1.5%; and 3.0%, respectively [4]. In humans, BoNT/A, B, and E were the primarily attributed poisoning toxins. Upon entering the neuron, the specific binding between the heavy chain of toxin and neuronal receptor rapidly occurs to cleave the light chain (LC) into the cytoplasm. Afterward, LC works as a protease that will enzymatic SNARE protein complex in the neuron thus inhibiting acetylcholine conduction [5].

Currently, a promising approach based on the next-generation nanobody was developed to enhance the recognition as well as inhibition of BoNT activity [6, 7]. Based on previous studies, two nanobodies, ALc-B8 (A8) and JNE-B10 (J10), have been selected for recombinant expression. These two nanobodies have been shown to have high-affinity and specificity binding to LC of BoNT/A and LC BoNT/B [5, 6, 8, 9]. The ability to synthesize nanobodies with known sequences facilitates the efficient production of recombinant antibodies in aqueous environments, contributing to applications in 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 systhesized by GenScript Biotech (USA). BoNT/A recombinant antigen was provided by the Vietnamese-Russian Tropical Hospital.

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℃ for about 10 - 20 minutes. Next, 120 ng of pET28a-A8/J10 plasmid is gently mixed with cells and incubated on ice for 20 minutes. The mixture is heat-shocked at 42℃ for 60 seconds, then transferred to ice for 5 minutes. Afterward, 500 - 700 µL of antibiotic-free LB medium was added to the mixture. The mixture then incubated at 37℃ with shaking at 150 rpm for 45 - 50 minutes. Cells were collected by centrifugation at 2300 rpm for 10 minutes and then cultured in a solid LB medium supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. Agar plates are cultured overnight at 37℃.

2.3.2. Determination of the presence of commercial plasmids by PCR technique

The plasmid was transformed into *E. coli* strains. To confirm the successful transformation of plasmid into cell strains, random colonies were selected tocolony PCR. Colony PCR and plasmid isolation from colonies was conducted using the vector primer pair Fw-T7 (5' TAATACGACTCACTATAGGG 3') and Rv-T7 (5' GCTAGTTATTGCTCAGCGG 3')/ The expected amplicon was 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 cultured in a liquid LB medium containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol and incubated at 37℃. Once the optical density at 600 nm (OD_{600}) reached a ranging of 0.5 to 0.8, Isopropyl (-D-1-thiogalactopyranoside (IPTG) was added at concentrations varying from 0 to 1 mM and continued to be cultured at 16℃ for 18 hours. Cell biomass was collected by centrifugation at 10000 rpm for 10 minutes and stored at -20℃.

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 and then sonicated on ice (4 cycles of 30 sec each). The mixture was centrifuged at 10000 rpm for 10 minutes at 4℃ to collect the supernatant as the total protein extract solution. The expression levels of the A8 and J10 nanobodies will be analyzed by SDS-PAGE electrophoresis and Western blotting.

2.3.4. Clean A8 and J10 nanobodies

The protein mixture was loaded onto the Ni-NTA affinity chromatography column, the collected solution was known as the flow- through (Ft) fraction 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 eliminate the bound protein forms the column. After the purification, the A8 and J10 nanobodies are applied through the PD10 desalting column (Cytiva Sephadex G-25) to remove residual imidazole. Finally, the concentration of protein was determined by measuring a adsorption at 280 nm.

2.3.5. Protein Electrophoresis on Polyacrylamide Gel with SDS (SDS-PAGE) [10]

Protein electrophoresis was conducted using SDS-PAGE following Laemmli's method [10]. The sample was prepared by mixing the protein solution with sample buffer containing 032 mM Tris base, 50% glycerol, 17 mM SDS, 2% mercaptoethanol, bromphenol blue and then heat treated at 95℃ for 5 minutes and subsenquantly cooled on ice. After the electrophoresis, the gel was stained with a 2.5% CBB solution (w/v). Destaining was carried out using methanol- and acetic acid - containing solution to remove the CBB until a clear background was achieved.

2.3.6. Dot bloting technique

LC-BoNT/A, B antigens were dropped onto the nitrocellulose membrane. Next, the membrane was incubated with A8 or J10 nanobodies as the primary antibody with varying concentration (0, 50, 100, 200 µg/mL). The HRP-binding Anti-Llama (Abcam, Cat. ab112786) was used as the secondary antibody to detect the affinity binding.

2.3.7. Western bloting technique

In the study, the aims of Western blotting expeiments were confirmation of the presense of A8, J10 and assessment of their immunoreactivity. For detection of nanobody expression, nanobodies A8 and J10 were performed on SDS-PAGE gel and transferred to nitrocellulose membranes. Afterward, the membrane was incubated with the primary antibodies, which were anti-His-tag monoclonal antibodies (mouse anti-histidine), and the secondary antibodies, which were AP-attached anti-mouse (akaline phosphatase).

For the evaluation of the immunoreactivity of A8, J10 nanobodies, LC-BoNT/A, B antigens were performed by electrophoresis and transferred to the nitrocellulose membrane which then incubated with the nanobody A8 /J10 as the primary antibody. The second antibody used was HRP-conjugated Anti-Llama (Abcam, Cat. ab112786). For control group, two commercial antibodies named 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) was used instead of nanobody.

2.3.8. ELISA technique

LC-BoNT/A, B antigens were coated on the 96-wells plate overnight. The coated plate then incubated with A8/J10 nanobodies as the primary antibodies. Afterward, the sandwich ELISA was continuously performed by adding HRP-binding Anti-Illama (Abcam, Cat. ab112786) as the secondary antibody.

3. RESULTS AND DISCUSSION

3.1. Evaluation of the expression level of A8, J10 nanobodies

The pET28a-A8 and pET28a-J10 plasmid were successfully transformed into *E. coli* BL21 (DE3) RIL bacteria and the colonies were tested by PCR reaction with vector-specific primer. 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 indicates that the plasmid has been successfully transduced into *E.coli* BL21 (DE3) RIL bacteria.

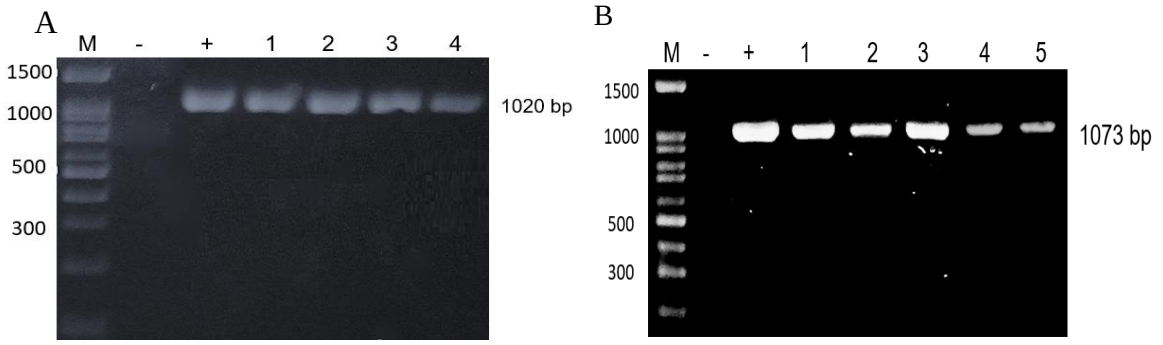

Figure 1. *Results of plasmid loading into BL21 (DE3) RIL with PCR product using Fw-T7 promoter/Rv-T7 terminator primer pair; PCR product electrophoresis images from colonies 1-4 of nanobody A8 (A) and colonies 1-5 of nanobody J10 (B). M: DNA Ladder 100 bp; (-): H2O negative control; (+): Plasmids are synthesized prior to loading*

After successful transformation, E.coli BL21 (DE3) RIL bacteria were cultured to induce with varying IPTG concentrations and collect the biomass for optimization and investigation the expersion level of A8/J10 nanobodies. This experiment was conducted at 16°C, with the IPTG's range from 0 mM to 1 mM. The SDS-PAGE gel showed the strongly overexpressed protein band at 33 kDa and 32 kDa, which are equivalent to the A8 and J10 nanobodies, respectively. These results suggested that the protein expression was most efficient at 0.5 mM IPTG treatment (**Figure 2**), which is consistent with the IPTG effective concentration 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 A8 nanobodies were distributed in the supernatant fraction, the J10 nanobodies were equivalently expressed in both of 2 fractions. However, the expression level of J10 nanobodies in supernatant fraction was sufficient for subsequent experiments.

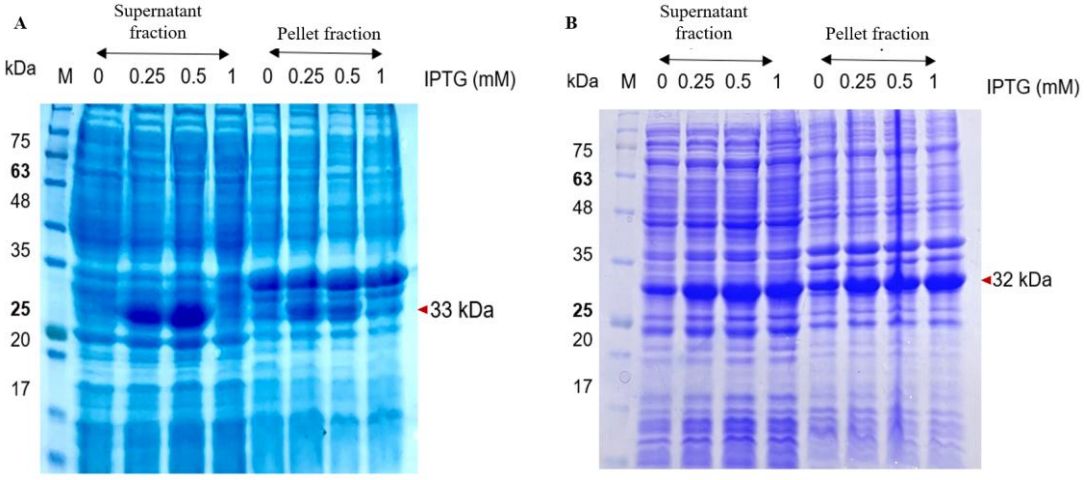

Figure 2. *A8 and J10 nanobody expression results at different IPTG concentrations. Note: A8 (A) and J10 (B) expression results were shown on SDS-PAGE gels. The red arrow marked the similar-sized location of the protein of interest. M: Protein Ladder*

3.2. Purification of A8 and J10 nanobodies

The A8 and J10 proteins, which contained His-tags, were extracted from E. coli and purified by Ni-NTA-Sepharose columns. The purification results showed a high recovery with 10 - 20 mg protein purified per liter of culture. The expression of nanobodies was further evaluated by Western blotting, utilizing a primary anti-histidine antibody to detect the His-tags specifically. The observed protein bands corresponded to the expected molecular weights of approximately 33 kDa for A8 and 32 kDa for J10, confirming the successful expression and purification of the A8 and J10 nanobodies (**Figure 3**).

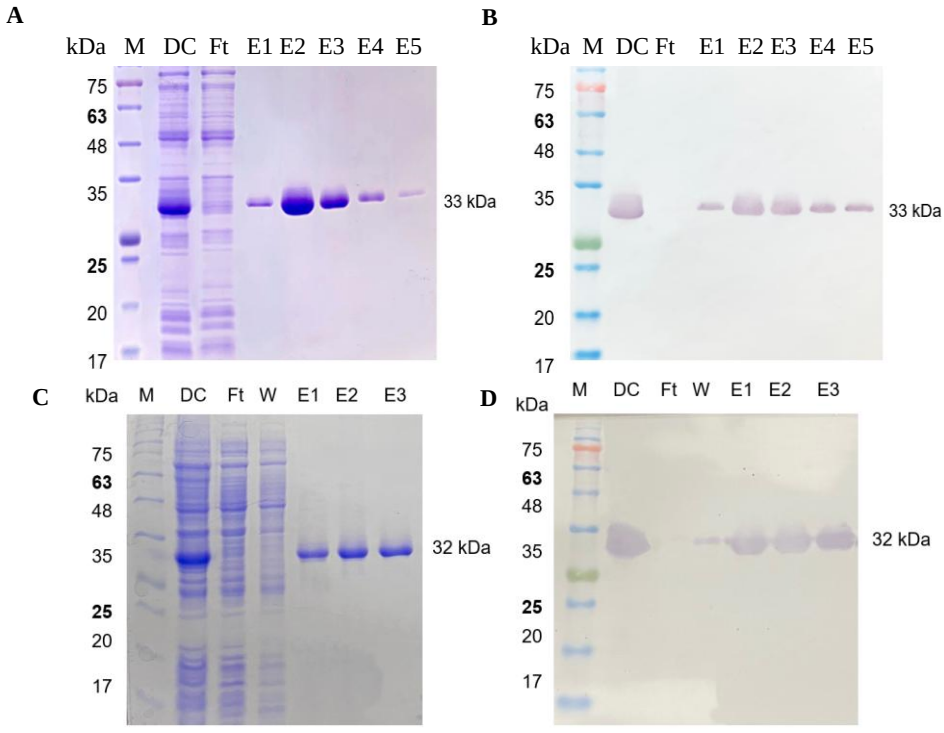

***Figure 3**. A8 and J10 nanobody purification results*

*The A8 nanobody purification results were shown on the SDS-PAGE (A) and evaluated by the Western blotting method (B**).** The results of J10 nanobody purification are shown on SDS-PAGE (C) and evaluated by Western blotting method (D). M: Protein ladder; DC: protein extract; Ft: segment without column; W: wash pad; E1, E2, E3, E4, E5: column-mounted segments 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 was further desalted by PD-10 columns and tested the immunoreactivity with BoNT/A and BoNT/B recombinant antigens. To determine the appropriate dilution of nanobodies before the immunoreactivity assay, the dot bloting was conducted.

As the results shown in **Figure 4**, only A8 nanobodies at 0 - 50 - 100 - 200 µg/mL was applied on dot bloting containing 0.3 µg BoNT/A recombinant antigen. The BoNT/A recombinant antigen optimictic detected at 100 µg/mL of A8 nanobody Based on this suggestion, the working concentration of J10 nanobody in immunoreactivity assay was also 100 µg/mL. Although this concentration was 200 - 500 folds higher than the concentration of commercial antibodies, the affinity of nanobodies may improve after removing the marker tag.

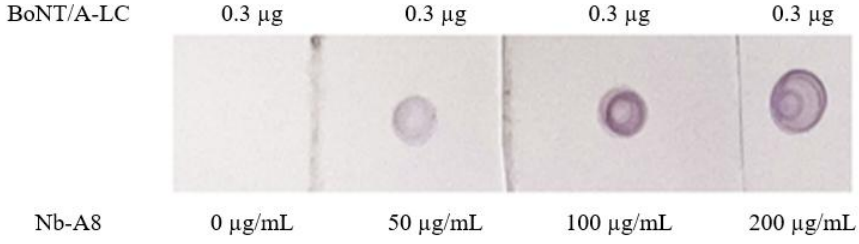

***Figure 4.** Determination of the nanobody concentration of the A8 nanobody*

LC-BoNT concentration of 0.3 µg is adsorbed onto the nitrocellulose film. The film was incubated with A8 nanobodies at different concentrations

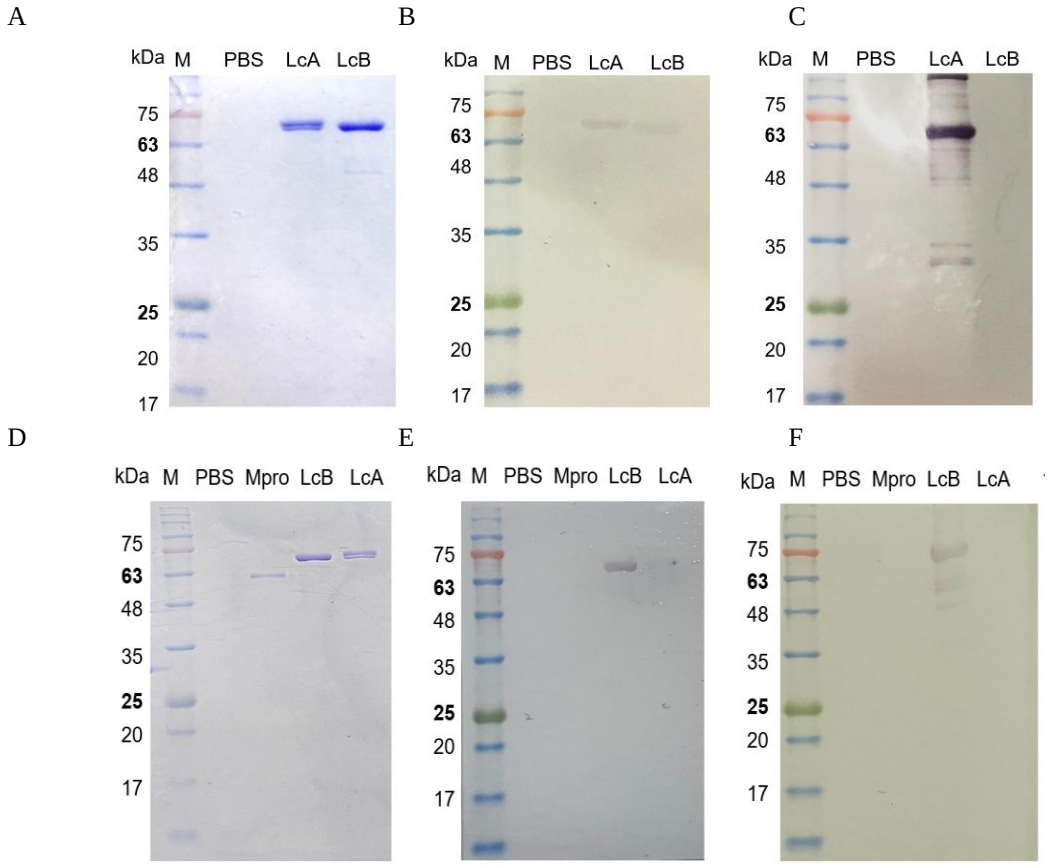

Figure 5. *Immunoreactivity assay of A8 and J10 nanobodies by Western bloting method (Recombinant antigens LC-BoNT/A, B on SDS-PAGE (A, D). The results of the LC-BoNT/A specific binding ability of nanobodies A8 (B) and commercial antibodies (C). The results of the LC-BoNT/B specific binding ability of the J10 nanobody (E) and commercial antibody (F). M: protein ladder, negative control: PBS, Mpro; LcA: LC-BoNT/A; LcB: LC-BoNT/B)*

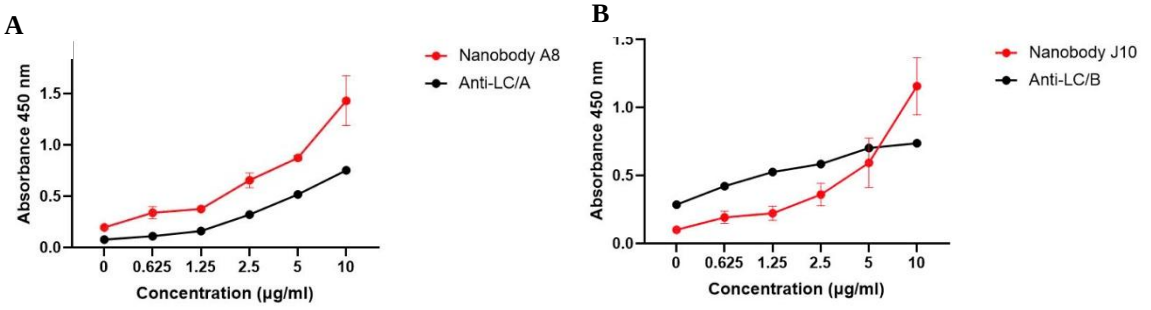

Figure 6. *Immunoreacitity assay of A8 and J10 Nanobodies by ELISA Method (LC-BoNT/A, B antigens with different concentrations are tested with nanobodies A8 (red line) and commercial antibodies (black lines) (A) or with nanobodies J10 (red lines) and commercial antibodies (black lines) (B). The secondary antibody used is Anti-Llama, which attaches HRP to the nanobody. Anti-LC/A, anti-LC/B: recognized commercial antibodies BoNT/A and BoNT/B, respectively)*

Western blotting results showed that both nanobodies A8 and J10 recognized BoNT/A and BoNT/B recombinant antigens through the presence of recombinant BoNT bands expected to be 70 kDa with positive controls being commercial antibodies Anti-LC-BoNT/A and Anti-LC-BoNT/B (**Figure 5**). In addition, the results also showed that the A8 nanobody, in addition to recognizing the detection of recombinant LC-BoNT/A antigens, had cross-recognition with LC-BoNT/B (20%) (**Figure 5B**), but no cross-binding was observed in

the J10 nanobody (**Figure 5E**). The ELISA results also showed the ability to recognize the BoNT/A antigen of A8 (**Figure 6A**) and the LC-BoNT/B recognition of J10 (**Figure 6B**). The ELISA signal increases as the concentration of the antigen increases, the strongest signal was recorded at 10 µg/mL antigen and 10 µg/mL nanobody.

Several studies have used A8 and J10 nanobodies for applications such as toxin neutralization and evaluating antigen-binding structures [6, 8]. However, these studies do not directly present the expression results as well as the specific antigen-binding interaction. Moreover, 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 at the N-terminal. To date, no publications have investigated the immunoreactivity of these two nanobodies in the fusion form. Therefore, this study contributed that the A8 and J10 nanobodies remain functional in their fusion form, which offers advantages in the antigen-binding assay by simplifying the experiment process. However, further investigations are required to compare the activity of the fusion form and the original form is needed to confirm which platform is the optimal performance.

The development of single-domain nanobody antibodies reveals high specificity forward targeted antigens, making them suitable for various applications. The toxin neutralization effect of A8 nanobody has been demonstrated in structural studies as well as other studies [6, 8], that means A8 nanobodies have the potential to bind to the active site of the BoNT/A-C enzyme, which was the main principle to detect botulinum toxin [6]. In contrast, J10 binds to an epitope region, which is not the center of activity of the BoNT/A-LC enzyme, making it suitable for toxin detection applications.

For toxin detection, both A8 and J10 antibodies could be used in the development of biosensors or lateral flow assays. In addition, these nanobodies can be conjugated to the magnetic particle to concentrate and separate BoNT from the complex food substrate, thereby enhancing the detection of toxins. Some studies have shown that the small size of nanobodies relates to challenges in conjugating on material surfaces, such as ELISA plates, magnetic particles, biosensors, etc. [11]. Therefore, increasing the size of the nanobody is an important strategy to conjugate nanobody on the material surface. Our study shows that the activity of nanobodies was maintained in fusion form with other proteins such as TrxA, suggesting the potential for the fusion of these nanobodies for immobilization of these nanobodies to the surface of materials for BoNT antigen recognition purposes.

4. CONCLUSION

Nanobodies A8 and J10 were successfully expressed on *E. coli* BL21 (DE3) RIL under 0.5 mM IPTG induction for 16 -18 hours at 16℃. Nanobody A8 and nanobody J10 were purified with high purity and an efficiency of about 10-20 mg/L of culture medium via Ni-Sepharose purification. The optimal concentration of the A8 and J10 nanobodies used to recognize the respective antigens in dot bloting and Western bloting is 100 µg/mL. The best ELISA signal of the nanobody to recognize 10 µg/mL of antigen is 10 µg/mL, respectively.

ACKNOWLEDGMENTS

This study was funded by the project VINIF, with the code VINIF.2022.DA00116 and the project KLEPT22.03. The research team would like to thank Dr. Le Thi Thu Hong donated the PD-10 column to the initial stage group to purify the protein.

REFERENCES

[1]. J. P. Salvador, L. Vilaplana, and M. P. Marco, "Nanobody: outstanding features for diagnostic and therapeutic applications," *Analytical and Bioanalytical Chemistry*, vol. 411, no. 9, pp. 1703-1713, 2019, doi: 10.1007/s00216-019-01633-4.

[2]. S. S. Arnon, R. Schecter, T. V. Inglesby, *et al.*, "Botulinum toxin as a biological weapon: Medical and public health management," *Journal of the American Medical Association*, vol. 285, no. 8, 2001, pp. 1059-1070, doi: 10.1001/jama.285.8.1059.

[3]. G. Yao, K. H. Lam, J. Weisemann, *et al.*, "A camelid single-domain antibody neutralizes botulinum neurotoxin A by blocking host receptor binding," *Scientific Reports*, vol. 7, no. 1, pp. 7438, 2017, doi: 10.1038/s41598-017-07457-5.

[4]. D. Kumaran, R. Rawat, S. A. Ahmed, and S. Swaminathan, "Substrate binding mode and its implication on drug design for botulinum neurotoxin A," *PLOS Pathogens*, vol. 4, no. 9, 2008, doi: 10.1371/journal.ppat.1000165.

[5]. J. M. Tremblay, C. L. Kuo, C. Abeijon, *et al.*, "Camelid single domain antibodies (VHHs) as neuronal cell intrabody binding agents and inhibitors of Clostridium botulinum neurotoxin (BoNT) proteases," *Journal of the International Society on Toxinology*, vol. 56, no. 6, pp. 990-998, 2010, doi: 10.1016/j.toxicon.2010.07.003.

[6]. S. I. Miyashita, J. Zhang, S. Zhang, C. B. Shoemaker, and M. Dong, "Delivery of single-domain antibodies into neurons using a chimeric toxin–based platform is therapeutic in mouse models of botulism," *Science Translational Medicine*, vol. 13, no. 575, 2021, doi: 10.1126/SCITRANSLMED.AAZ4197.

[7]. P. M. Mcnutt, E. J. Vazquez-Cintron, L. Tenezaca, *et al.*, "Neuronal delivery of antibodies has therapeutic effects in animal models of botulism," *Science Translational Medicine*, vol. 13, no. 575, 2021, doi:10.1126/scitranslmed.abd7789.

[8]. K. ho Lam, J. M. Tremblay, E. Vazquez-Cintron, *et al.*, "Structural Insights into Rational Design of Single-Domain Antibody-Based Antitoxins against Botulinum Neurotoxins," *Cell Reports*, vol. 30, no. 8, pp. 2526-2539, 2020, doi: 10.1016/j.celrep.2020.01.107.

[9]. K. H. Lam, J. M. Tremblay, K. Perry, K. Ichtchenko, C. B. Shoemaker, and R. Jin, "Probing the structure and function of the protease domain of botulinum neurotoxins using single-domain antibodies," *PLOS Pathogens*, vol. 18, no. 1, 2022, doi: 10.1371/journal.ppat.1010169.

[10]. U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature*, vol. 227, no. 5259, pp. 680-685, 1970, doi: 10.1038/227680a0.

[11]. G. C. Girt, A. Lakshminarayanan, J. Huo, *et al.*, "The use of nanobodies in a sensitive ELISA test for SARS-CoV-2 Spike 1 protein," *Royal Society Open Science*, vol. 8, no. 9, pp. 211016, 2021, doi: 10.1098/rsos.211016.