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Recombinant Anti-Botulinum Neurotoxin A Single-Chain Variable Fragment Antibody Generated Using a Phage Display System

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ABSTRACT

A recombinant single-chain fragment variable antibody (scFv) to botulinum A neurotoxin (BoNT/A) was developed. BALB/C mice were immunized with BoNT/A. Splenomic RNA was isolated from the hyperimmune mice and used to prepare a cDNA library, from which the variable regions of the heavy and light chain antibody genes were generated and connected by a DNA linker. The resulting scFv genes were cloned into the phagemid vector pCANTAB5 in order to construct phage display scFv libraries. Individual anti-BoNT/A phage clones were isolated from the phage display libraries by immunoaffinity selection using immobilized BoNT/A and further evaluated by enzyme-linked immunosorbant assay, immunoprecipitation and Western blotting. Forty-eight clones were found to be BoNT/A-reactive. The most reactive clone, designated D12, was selected for further study. The scFv gene of D12 was subcloned into a *Pichia pastoris* vector, and expression in yeast was evaluated.

INTRODUCTION

BOTULINUM NEUROTOXINS (BoNT) produced by strains of *Clostridium botulinum*, are the most lethal toxins known affecting humans and other mammals.[1,2] Since BoNT is so lethal and easy to manufacture, deliver, and weaponize, it is a threat as a toxin warfare agent.

Seven serotypes of BoNT, classified A to G, are recognized. All seven subtypes act by similar mechanisms. BoNT bind to the presynaptic nerve terminal at the neuromuscular junction and cholinergic autonomic sites and thus disrupt cellular communication at the neuromuscular junction, resulting in muscular weakness and paralysis.[3] Serotype A can be purified consisting of a toxic component, botulinum neurotoxin serotype A (BoNT/A), and a non-toxic component, hemagglutinin.[4–6]

Neutralizing monoclonal antibodies to BoNT/A could provide an efficient passive immunity to endangered individuals. However, the disadvantages of using murine monoclonal antibodies as immunotherapeuticagents in humans are apparent.[7,8] The biggest one is human anti-mouse antibody responses.[8] Limitations in the use of murine monoclonal antibodies in clinical applications boosted the development of single-chain fragment variable antibodies (scFv), in which the variable region of heavy chain (VH) and the variable region of light chain (VL) of the antibody are linked by a peptide.[9] The scFv showed similar affinity to antigens. Most importantly, since scFv comprise about 25 kDa as compared to the 150 kDa of an intact IgG molecule, the low antigenicity of scFv makes the molecule an attractive alternative to whole mouse antibodies as therapeutic agents[10] and may be better able to cross the blood–brain barrier, to effect a treatment.

In this study, we constructed phage display scFv libraries (10^5–10^6 independent clones) by cloning scFv genes from mRNA derived from BoNT/A-immunized mice into pCANTAB5 vectors, selected the anti-BoNT/A scFv phage clones by screening the phage display libraries with BoNT/A via three rounds of biopanning, further evaluated anti-BoNT/A scFv phage clones by enzyme-linked immunosorbent assay (ELISA), immunoprecipitation and Western blotting. Forty-eight clones were found to be anti-BoNT/A reactive. The most

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reactive clone, designated D12, was selected for expression in yeast.

MATERIALS AND METHODS

BoNT/A and BoNT/A toxoid (BoTd/A)

BoNT/A was obtained from Wako Chemicals (Richmond, VA). BoNT/A toxoid (BoTd/A) was prepared by incubating BoNT/A in 3% formaldehyde at 37°C for 10–14 days and then dialyzing extensively against phosphate buffered saline (PBS), pH 7.2. Highly purified (99% pure) BoNT/A was obtained from the Food Research Institute, University of Wisconsin (Madison, WI).

Immunization of mice

Three-week-old BALB/C female mice were immunized with BoNT/A using a two-phase immunization schedule. All experiments were approved by DRDC Suffield animal care committee. In phase one, the mice received five intraperitoneal (i.p.) inoculations of 20 μg of BoTd/A supplemented with 20 μg of muramyl dipeptide adjuvant at 10–14-day intervals. In phase two, mice received four i.p. inoculations of increasing amounts of BoNT/A from 10 ng (300 LD_{50}) to 5 μg, supplemented with 20 μg of muramyl dipeptide adjuvant at the same intervals as in phase one. No muramyl dipeptide adjuvant was added in the final booster. The mice were sacrificed and their spleens were removed approximately 10 days after the final inoculation. Antiserum titers at that time were between 1:1,000 and 1:10,000, based on immunodot blots using biotinylated BoNT/A.

Construction of phage display libraries

Four spleens were pooled for each of two extractions. The tissues were homogenized in a solution of guanidinium isothiocyanate, and RNA was further purified by isopycnic density gradient centrifugation on cesium chloride.[11] The RNA pellet was resuspended in nuclease-free water, and stored precipitated in ethanol. Yields of 600–650 μg of splenic total RNA were obtained.

Splenic total RNA (10–20 μg per reaction) was used as template for reverse transcription. Primer mixtures from the Recombinant Phage Antibody System–Cloning Kit (Amersham Pharmacia Biotech, Baie d' Urfe', QC) were then used in polymerase chain reaction (PCR) (94°C/1 min; 55°C/2 min; 72°C/2 min; 30 cycles) to amplify cDNA sequences corresponding to VH and VL of mouse IgG. DNA fragments were resolved by electrophoresis in 0.8% agarose and purified by phenol/chloroform extraction followed by ethanol precipitation. Equal amounts (0.1–0.2 μg) of gel-purified VH and VL DNA were annealed in-frame with a $(Gly_4Ser)_3$ linker–primer. Full-length scFv gene fragments were then generated using *Taq* DNA polymerase (94°C/1 min; 63°C/4 min; 7 cycles). Buffer conditions were optimized and full-length scFv DNA fragments were amplified by PCR (94°C/1 min; 55°C/2 min; 72°C/2 min; 30 cycles) using 5' and 3' primers incorporating *Sfi*I and *Not*I restriction sites, respectively. The scFv DNA fragments were gel purified, and digested with *Sfi*I and *Not*I. The restriction enzyme-digested fragments were gel purified a second time and ligated into the phagemid pCANTAB 5 at the *Sfi*I and *Not*I sites.

Competent *E. coli* TG1 cells were transformed with the ligated DNA (5–50 μL) by the method of Hanahan.[12] Phage rescue with M13KO7 helper phage (Amersham Pharmacia Biotech) resulted in the production of recombinant phage containing copy of the phagemid DNA encoding an scFv gene. The recombinant phage would display one or more copies of the scFv as fusion protein linked to the phage glycoprotein, gp3. For phage rescue, 100 μL of an overnight culture of the transformed TG1 was added to 1 mL of 2 × YT broth (16 g/L bactotryptone, 5 g/L yeast extract, 10 g/L NaCl, and 25 mM Tris-HCl, pH 7.5) supplemented with 100 μg/mL ampicillin, 2% glucose and 3×10^{10} pfu/mL M13KO7. This culture was incubated at 37°C for 2 h, with shaking at 250 rpm. The cells were pelleted by centrifugation for 10 min at 5,000 × *g* and the supernatant was discarded. The cell pellet was resuspended in 5 mL of 2 × YT broth supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin, and incubated overnight at 37°C, with shaking at 250 rpm. The cells were pelleted by centrifugation for 15 min at 5,000 × *g*, and the recombinant phage display libraries were collected in the supernatant.

Selection of recombinant anti-BoNT/A scFv phage clones

All procedures using BoNT/A were carried out in a biosafety cabinet or fumehood by personnel immunized to BoNT. This process ultimately resulted in enrichment for those phage clones, which displayed antigen-specific scFv on their surfaces. Affinity selection was conducted in Falcon polyvinyl carbonate (PVC) 96-well round-bottom plate (Fisher Scientific, Nepean, ON). Fifty microliters of BoNT/A antigen (6 μg/mL) was added to each well containing 50 μL of 2 × coating buffer (0.1 M sodium carbonate, pH 9.2). The plate was incubated overnight at 4°C. Unbound antigen was removed from the wells by two rinses with sterile PBS containing 0.05% Tween 20 (PBST). One hundred and fifty microliters of blocking buffer (sterile 5% skim milk in PBS) was added to the well. The plate was incubated for 2 h at room temperature. The phage library was pre-adsorbed, by adding 150 μL of the phage into the blocked well, which contained no antigen and incubated for 1 h at room temperature. Following the pre-adsorption step, the phage library was transferred to the well containing bound antigen. The plate was incubated for 1 h at room temperature, after which, unbound phage were removed from the well. The well was then rinsed ten times with PBST and ten times with PBS. Bound phage were eluted by the addition of 150 μL of 100 mM triethylamine and transferred to a microfuge tube and neutralized with 75 μL of sterile 1 M Tris, pH 7.2. After four consecutive above-described affinity selection cycles, 200 μL of *E. coli* TG1 overnight culture was added to a 5-mL tube containing 20 μL of eluted phage. The tube was incubated at 37°C for 30 min, with shaking at 150 rpm. The content was then spread onto Luria-Bertani (LB) agar plates supplemented with 100 μg/mL ampicillin and 2% glucose; and incubated at 37°C overnight. Single colonies from plate were inoculated into 1.5 mL of LB broth supplemented with 100 μg/mL ampicillin and 2% glucose and incubated at 37°C overnight. Phagemid was rescued with M13KO7 helper phage as described above.

Evaluation of selected phage clones by ELISA, immunoprecipitation, and Western blotting

An ELISA, designed to detect anti-BoNT/A scFv phage clones, was conducted to determine which of the individual recombinant phage clone displayed anti-BoNT/A scFv as previously described.[13] Fifty microliters of 6 μg/mL BoNT/A was coated on to Falcon PVC 96-well round-bottom plates. Two wells were used as negative controls; one well received 50 μl PBS and the other well received 50 μL of 10 μg/mL BSA. One well received 50 μL of M13KO7 phage at a concentration of greater than 5×10^{10} pfu/mL as a positive control. The wash buffer was PBST, and the blocking buffer was 5% skim milk in PBST. After blocking, individual phage from select clones were added to separate wells, 120 μL/well. The plate was incubated at room temperature for 1 h, followed by washing with PBST. Sheep anti-M13 horseradish peroxidase (HRP) conjugate (Amersham Pharmacia Biotech) diluted 1:2,000 in blocking buffer was added to the wells, incubated at room temperature for 1 h, and the plate was rinsed once with PBST and once with PBS. Substrate [2,2'-azino-bis(3-ethybenzthiazoline-6-sulfonic acid)] (ABTS) and hydrogen peroxidate (Kirkegaard and Perry Laboratories, Gathersburg, MD) was added, the plate was incubated at room temperature for 1 h, and monitored for color development.

Clones which appeared to produce the desired scFv, based on ELISA, were further tested for their ability to immunoprecipitate biotinylated BoNT/A. BoNT/A was biotinylated in accordance with procedures provided with a biotinylation kit purchased from Amersham Pharmacia Biotech. Selected recombinant phage were incubated with biotinylated BoNT/A at room temperature for 1 h, and then with streptavidin-coated beads (Pierce, Rockford, IL) for 1 h. Immunoprecipitates were collected by centrifugation and washed three times with 0.5% Nonidet-EDTA (NET) (Sigma, Oakville, ON) buffer. Bound proteins were released from the precipitate by the addition of 2 × Laemmli Sample Buffer (Bio-Rad, Mississauga, ON). Samples of the released proteins were analyzed by electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels. The separated proteins were transferred by a western blot semi-dry transfer apparatus (Bio-Rad) onto Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked, and then exposed to sheep anti-M13 HRP conjugate. Bands were visualized using an enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech).

Meanwhile, ELISA-positive phage clones were examined by a second method for their ability to immunoprecipitate biotinylated BoNT/A. Selected phage were incubated with biotinylated BoNT/A at room temperature for 1 h, then with rabbit anti-M13 antibodies (Stratagene, La Jolla, CA) for 1 h, and finally with beads bearing immobilized protein G (Amersham Pharmacia Biotech). Immunoprecipitates were collected and washed as described above. Bound proteins were released and analyzed as above, except that streptavidin-alkaline phosphatase (AP) conjugate (Pierce) was used instead of sheep anti-M13 HRP conjugate. ECL detection was performed using CSPD^R substrate (Bio/Can, Mississauga, ON).

Western blotting was performed as previously described to confirm above-selected phage clones to bind to BoNT/A. The BoNT/A antigen was separated by 10% SDS-PAGE gel and then transferred on to Immobilon-P membrane using a western blot semi-dry transfer apparatus. The membrane was blocked in blocking buffer (2% BSA in PBS) at room temperature for 2 h, and then incubated at room temperature for 1 h with 2 mL of selected phage clones in blocking buffer. The membrane was then rinsed several times with PBST and incubated at room temperature for 1 h in a 1:2,000 dilution of rabbit anti-M13 HRP conjugate in blocking buffer. After rinsing several times with PBST and then twice with PBS, the specific binding was detected by ECL kit.

DNA sequencing

Phagemid DNA from clone D12 was chosen for DNA sequencing using a pCANTAB 5 sequencing primer set from Amersham Pharmacia Biotech. The sequencing reactions were performed using a Big Dye Terminator™ cycle sequencing kit (PE Biosystems, Mississauga, ON). The reaction products were purified by Centri-Sep™ columns (Princeton Separations, Adelphia, NJ), and then run on an ABI 310 genetic analyzer system (Applied Biosystem, Fullerton, CA). Sequences were assembled and analyzed using Lasegene DNA software (DNA star, Madison, WI). Comparison of DNA sequence to Genbank was performed with IgBLAST through the internet at www.ncbi.nlm.nih.gov/igblast/.

Expression of D12 scFv in yeast

After above-described selection, evaluation, and DNA sequence analysis, the most reactive phage clone (D12) scFv gene was subcloned into a yeast expression vector pPIC9 (Invitrogen, Burlington, ON). In order to easily detect and purify expressed scFv, a 6His tag was introduced to the expression vector. The vector pPIC9H6 was first constructed as follows. The gene fragment encoding the 6His-enterokinase cleavage site (EK) domain in pRSET B (Invitrogen) was amplified by PCR using a pair of primers (one containing a *EcoR*I site) and the amplified product was digested with *EcoR*I. This 6His-EK fragment was then ligated into the *SnaB*I (blunt end) and *EcoR*I sites of pPIC9 plasmid, downstream of *Saccharomyces cerevisiae* α–factor secretion signal, thus producing vector pPIC9H6. A *EcoR*I D12 primer and S6 primer containing a *Not*I site were used to amplify D12 scFv gene fragment by PCR. The *EcoR*I- and *Not*I-digested D12 scFv fragment was then ligated into the *EcoR*I and *Not*I sites of pPIC9H6 plasmid, thus creating pH6D12 construct (Fig. 1). Successful cloning was confirmed by restriction analysis. The pH6D12 was linearized and transformed into *P. pastoris* strain GS115 competent yeast cells (Invitrogen), using either the spheroplast method or the lithium chloride method described in Invitrogen's manual. Expression was conducted following the Invitrogen protocols. Briefly, pH6D12-transformed *P. pastoris* was grown to mid-log phase in buffered glycerol-complex medium (BMGY) at 30°C, 250 rpm, and resuspended in buffered methanol-complex medium (BMMY) (BMGY with 0.5% methanol), to grow for three more days. Supernatant and cells were collected for analysis. They were analyzed by SDS-PAGE, followed by Coomassie blue staining. Samples on a duplicate SDS-PAGE gel were transferred onto an Immobilon-P membrane using a western blot semi-dry transfer apparatus. Western blotting was performed using mouse

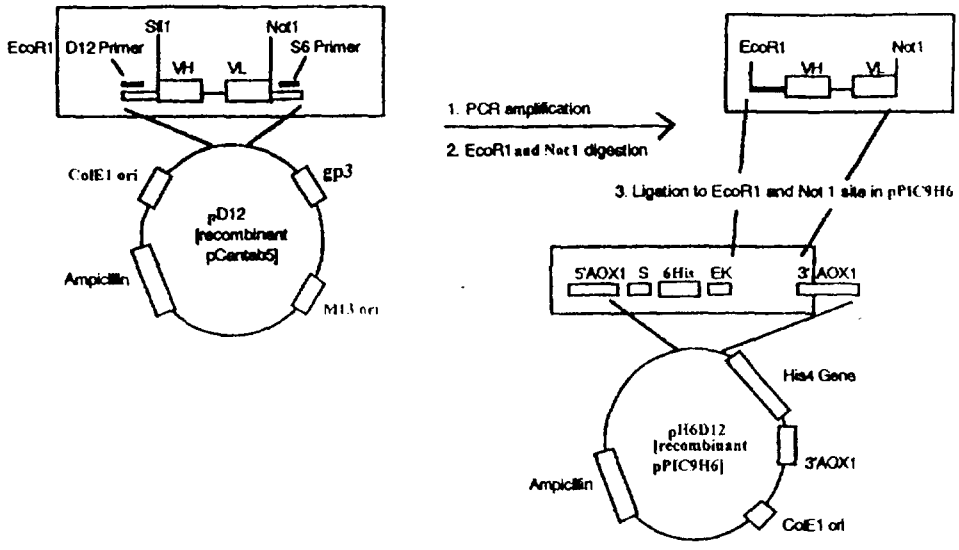

FIG. 1. Construction of pH6D12, pPIC-based yeast expression vector. The vector was designed to express scFv as a fusion product with the 6His-EK domain. D12 scFv gene was amplified by PCR. The *EcoRI*- and *NotI*-digested D12 scFv gene was then ligated into the *EcoRI* and *NotI* sites of pPIC9H6. AOX, alcohol oxidase gene; S, *Saccharomyces cerevisiae* α-factor secretion signal gene.

monoclonal antibody to the EK peptide domain (Invitrogen) (1:3,000), followed by donkey anti-mouse HRP (Caltag Laboratories, Burlingame, CA). The specific binding was detected by an ECL kit.

RESULTS

Construction of phage display libraries

Splenic total RNA from eight immunized BALB/C mice was reverse transcribed and the resulting cDNA was amplified by PCR, to generate distinct bands of ~300 bp V_H and V_L detected by agarose gel electrophoresis (Fig. 2). The VH and VL DNA fragments, joined through a $(Gly_4Ser)_3$ linker, were amplified by PCR to generate 750-bp scFv gene fragments (Fig. 2). The scFv DNA fragments were further amplified using primers containing *SfiI* and *NotI* sites. These products were then digested with *SfiI* and *NotI* and ligated to the corresponding sites in linearized pCANTAB 5 phagemid vector. Phagemid obtained by transformation of TG1 *E. coli* with ligated DNA were selected on LB medium containing glucose and ampicillin. Phage rescue with M13KO7 was then utilized to create phage display libraries. Thirteen such libraries, with complexities of 10^5 to 10^6, were obtained from six preparations of scFv DNA fragments.

Selection and evaluation of recombinant anti-BoNT/A scFv phage clones

M13 phage clones containing recombinant scFv genes and displaying scFv specific to BoNT/A were isolated by immunoaffinity selection (biopanning) using BoNT/A immobilized on the wells of PVC plates. Four thousand isolated clones were then screened by ELISA using BoNT/A (Wako Chemicals). Of these, 130 (3%) gave positive ELISA results. Ninety-six of the 130 clones were further screened by ELISA, using highly purified BoNT/A. Forty-eight of the 96 clones (50%) gave positive ELISA results (data not shown). The 29 most reactive clones were listed in Table 1.

Three methods were used to further evaluate the reactivity of the clones, which gave positive ELISA results. In the first method, selected clones were allowed to bind to biotinylated BoNT/A, and the immune complexes were precipitated with streptavidin-coated beads. Bound proteins were then released from the precipitate and analyzed by SDS-PAGE and Western blotting, using sheep anti-M13 HRP conjugate. Of 10 clones tested, all were reactive and six were highly reactive (Table 1). In the second method, selected clones were allowed to bind biotinylated BoNT/A, as described above. Rabbit anti-M13 antibodies were then added, followed by protein G-coated beads.

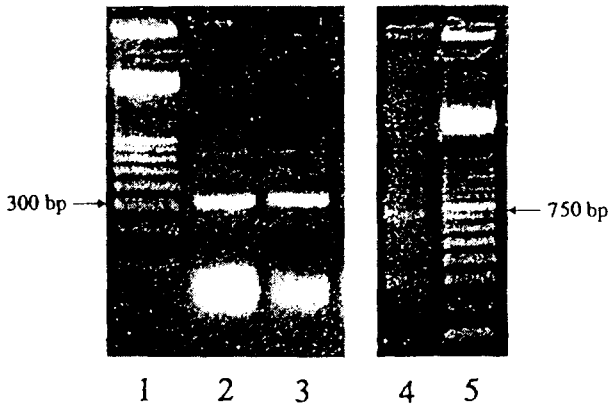

FIG. 2. Agarose gel electrophoresis of DNA fragments. Lanes 1 and 5, DNA markers; lanes 2, 3, PCR products of VH and VL; lane 4, scFv DNA fragments linked by the linker.

TABLE 1. SUMMARY OF ANTI-BoNT/A scFv PHAGE CLONES

Clone	*ELISA*[a]	*IP with M13*[b]	*IP with BoNT/A*[c]
A4	++	ND[c]	ND
A5	++	+	++
A7	+	++	+
A10	+	ND	++
B2	+	ND	++
B4	++	++	++
B7	+++	ND	ND
B8	++	ND	ND
B10	++	+	ND
C3	++	ND	ND
C10	++	++	ND
D1	+	+	++
D9	++	ND	ND
D10	+	++	++
D12	++	+++	+
E2	+++	ND	ND
E4	++	ND	ND
E6	++	++	++
E9	++	+	+
E10	++	ND	ND
E12	++	ND	ND
F5	++++	ND	ND
F9	++	ND	ND
F11	++	ND	ND
G1	+++	ND	+
G6	++	ND	ND
G12	++	ND	ND
H4	++	ND	ND
H7	++	ND	ND

[a]Relative intensity of positive ELISA signals of anti-BoNT/A scFv phage clones against highly purified BoNT/A.

[b]Relative intensity of positive ECL signals of immunoprecipitated anti-BoNT/A scFv phage clones using anti-M13 conjugate.

[c]Relative intensity of positive ECL signals of immunoprecipitated anti-BoNT/A scFv phage clones using streptavidin-AP conjugate.

Bound proteins were then released from the immunoprecipitated complexes and analyzed by SDS-PAGE and Western blotting analysis, using a streptavidin-AP conjugate. Of 11 clones tested, four were reactive and seven were highly reactive (Table 1). Thus, confirmation was obtained that the ELISA screening procedure identified clones, which did, in fact, produce anti-BoNT/A scFv. The confirmation methods were distinct. The first method relied on anti-M13 conjugate for detection of M13 phage. The second method was based on streptavidin-AP conjugate for detection of BoTN/A. The ability of the clones to bind to BoNT/A was also tested more directly by a third method, as follows. BoNT/A was subjected to SDS-PAGE and electroblotted onto the membrane. Finally, Western blotting was then performed, using selected recombinant M13 phage as primary antibody and rabbit anti-M13 HRP conjugate as secondary antibody. All clones tested showed binding to BoNT/A (data not shown).

DNA sequence analysis of D12

The DNA and deduced amino acid sequences of D12 scFv were shown in Figure 3. The complementary determinant regions (CDR) of VH and VL were indicated. The D12 scFv gene was 738 bp in length, encoding 246 residues with a molecular weight of 27 kDa. The frameworks for VH and VL regions were well conserved. The VH gene was most homologous to murine VOx-1 of murine Ig heavy germline V genes (90% identity). The heavy chain V gene segment was rearranged with a J gene segment homologous to murine JH1 gene (94% identity). The VL gene was most homologous to the murine Ig kappa germline gene, aq4 (95% identity), and the V gene segment was rearranged with a J gene segment homologous to murine JK1 (95% identity).

Expression of D12 scFv in Pichia pastoris

D12 scFv gene was subcloned into a yeast expression vector pIC9. In order to easily detect and purify expressed scFv antibody, a 6His tag was introduced to the expression vector. First, a yeast vector pPIC9H6 was constructed. D12 scFv gene fragment was then successfully subcloned into the plasmid pPIC9H6. The resulting plasmid, designated pH6D12 contained a DNA sequence encoding a peptide with the *Saccharomyces cerevisiae* α–factor secretion signal followed by the 6His-EK gene before the D12 scFv gene. The pH6D12 was transformed into *P. pastoris* strain GS115 using the spheroplast or lithium chloride method. These yeast clones were expected to express H6D12 (around 40 kDa). Western blotting analysis of medium supernatants and cellular extracts, using mAb to EK, indicated that H6D12 was expressed intracellularly (data not shown). Although some pH6D12 clones expressed extracellularly 30 kDa protein shown on SDS-PAGE (Fig. 4), they could not be detected by anti-EK in Western blotting (data not shown).

DISCUSSION

In this study, we demonstrated the validity of generating scFv against BoNT/A directly from immunized mice using a phage display system. The generation of recombinant mouse scFv relies on hyperimmunized mice. In order to get a strong anti-BoNT/A immunity in mice, two phases of immunization were performed. The first phase with BoTd/A immunization followed by the second phase with BoNT/A immunization. In this way, a high level of anti-BoNT/A in mice was obtained, indicating a large amount of anti-BoNT/A mRNA in mice splenic tissue and a solid foundation was laid to generate anti-BoNT/A ScFv successfully.

Due to limited availability of highly purified BoNT/A, low-purity BoNT/A was used in the initial immunoaffinity purification and ELISA of recombinant phage clones. When reactive clones were subsequently re-assayed using highly purified BoNT/A, it was determined that only 50% of them produced anti-BoNT/A scFv antibodies. The positive ELISA signals obtained using this highly purified BoNT/A were generally stronger than those obtained using the lower purity BoNT/A. Using this approach, we could get a high BoNT/A-specificity of phage clones by the limited amount of highly purified BoNT/A.

ELISA is the preferred methodology for many routine diagnostics because it is rapid and convenient. Accordingly, ELISA

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1   ATGGCCCAGGTCCAACTGCAGGAGTCAGGACCTGGCCTAATGGCGCCCTCACAGAGCCTGTCCATAACCTG
1▶  M  A  Q  V  Q  L  Q  E  S  G  P  G  L  M  A  P  S  Q  S  L  S  I  T  C

72  CACTGTCTCTGGATTCTCATTAACCAGCTATGATATAAGCTGGATTCGCCAGCCACCAGGAAAGGGTCTGG
24▶  T  V  S  G  F  S  L  T  S  Y  D  I  S  W  I  R  Q  P  P  G  K  G  L

143 AGTGGCTTGGAGTAATATGGACTGGTGGAGGCACAAATTATAATTCCGCTTTCGTGTCCAGACTGAGCATC
48▶ E  W  L  G  V  I  W  T  G  G  G  T  N  Y  N  S  A  F  V  S  R  L  S  I

214 AGCAAGGACAACTCCAAGGGCCAAGTTTTCTTAAAAATGAACAGTCTGCAAACTGATGACACAGCCATATA
72▶  S  K  D  N  S  K  G  Q  V  F  L  K  M  N  S  L  Q  T  D  D  T  A  I  Y

285 TTACTGTGTAAGAGATGACTATAATAACGACAGGTATGCTATGAACTATTGGGGGCATGGCACCACGGTCA
95▶  Y  C  V  R  D  D  Y  N  N  D  R  Y  A  M  N  Y  W  G  H  G  T  T  V

356 CCGTCTCCTCAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATCGA
119▶ T  V  S  S  G  G  G  G  S  G  G  G  G  S  G  G  G  G  S  D  I  E

420 GCTCACTCAGTCTCCAACACTCATGTCTGCATTTCTAGGGGAGAGGGTCACCATGGCCTGCAGTGCCAGCT
140▶  L  T  Q  S  P  T  L  M  S  A  F  L  G  E  R  V  T  M  A  C  S  A  S

491 CAAATCTAAGTTACATGAACTGGTACCAGCAGAGGCCAAGATCCTCCCCCAAACCCTGGATTTATCTCACA
164▶ S  N  L  S  Y  M  N  W  Y  Q  Q  R  P  R  S  S  P  K  P  W  I  Y  L  T

562 TCCAACCTGACTTCTGGAGTCCCTGCTCGCTTCAGTGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAAT
188▶ S  N  L  T  S  G  V  P  A  R  F  S  G  S  G  S  G  T  S  Y  S  L  T  I

633 CAGCAGCATGGAGGCTGAAGATGCTGCCACTTATTACTGCCAGCAGTGGAGTAGTAACCCATTCACGTTCG
211▶  S  S  M  E  A  E  D  A  A  T  Y  Y  C  Q  Q  W  S  S  N  P  F  T  F

704 GCTCGGGCATCAAGCTGGAAATCAAACGGGCGGCCGC
235▶ G  S  G  I  K  L  E  I  K  R  A  A
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FIG. 3. DNA sequence and deduced amino acid sequence of D12 scFv, composed of VH and VL connected by the linker (bold). The CDR regions for both VH and VL are underlined.

was used to evaluate reactive clones. The reliability of ELISA for the identification of the desired phage clones was confirmed using two distinct immunoprecipitation techniques, and further verified by a more direct Western blotting analysis.

DNA and deduced amino acid sequences of D12 scFv consisted of the conservative framework and three CDRs for either VH and VL. The gene families of VH and VL were elucidated. As expected, the CDRs for both VH and VL of scFv D12 gene showed great diversity as compared with CDRs of VHs and VLs of other monoclonal antibodies. The diversity of CDR sequences determines affinity and specificity of antibody-antigen binding.

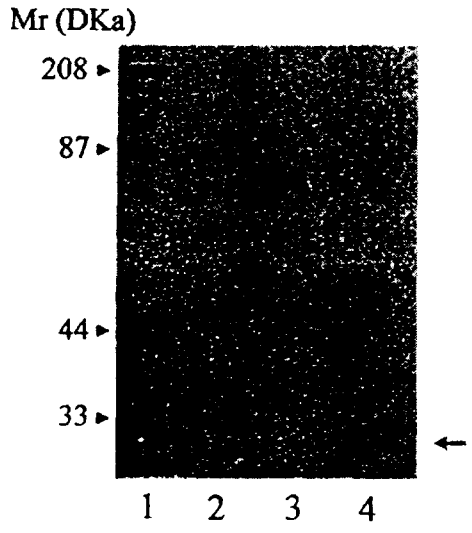

FIG. 4. SDS-PAGE analysis of GS115 yeast supernatants. Samples were resolved on 10% polyacrylamide gel and stained with Coomassie blue. Lane 1, molecular weight marker; lane 2, GS115; lane 3 GS115 harbouring the control plasmid encoding bovine serum albumin; lane 4, GS115 harbouring pH6D12.

After the successful selection of scFv phage clones specific for BoTN/A, the expression of soluble scFv was the major concern. The most frequently used prokaryotic vector system for the expression of scFv has been *E. coli*, in which the scFv are obtained by either in vitro refolding from inclusion bodies or by secretion to the bacterial periplasm.[13–15] Much effort has been made to improve the functional yields of scFv from *E. coli*. However, the methods require extensive optimization at purification levels with low yields of functional scFv from the periplasmic inclusion bodies. Furthermore, for clinical applications, the removal of bacterial endotoxins is essential.[16] A great advantage to the expression of scFv antibodies in *P. pastoris* is that the yields could probably be increased, due to the availability of strong promoters and the ability of the yeast to grow at extremely high cell densities.[17] The system also offers all the advantages of expression in a eukaryotic host, protein processing, folding, post-translational modifications and secretion to media. One more advantage is that *P. pastoris* secrete negligible amounts of native proteins. ScFv have been successfully expressed in yeast systems, for example, an anti-tumor associated antigen scFv[18] and anti-human CD18 scFv.[19] In our study, the H6D12 scFv was retained intracellularly, suggesting that the 6His-EK domain may interfere with the secretory processes and pathways of H6D12 scFv expressed in *P. pastoris*. Some H6D12 clones expressed secreted products of predicted size of D12 scFv and were probably deleted in the 6His-EK domain, as they could not be detected by Western blotting with anti-EK and supernatant was found to be able to prolong survival time of mice challenged with BoNT/A in a preliminary neutralization bioassay. Further studies will be conducted to switch 6His-EK domain from N-terminus to C-terminus of D12 in order to solve the problem of interference of

6His-EK in C-terminus with the secretory processes and pathways of H6D12 scFv expressed in *P. pastoris*.

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