11.2 Western blot analysis

In WB, crude Thai cobra venom was separated on SDS-PAGE (15% gel) under non-reducing condition, then was electro-blotted onto the NC, and probed with soluble HuScFv in whole cell lysate.

A. Cobra venom factor (P1) (**Figure 87**)

1. HuScFv of clones P1/1 and P1/5 reacted with protein bands at *Mr* > 195 and ~45 kDa.

2. HuScFv of clone P1/2 reacted weakly with protein bands at *Mr* > 195 and ~45 kDa.

3. HuScFv of clones P1/3 and P1/9 could not react with any protein band on the blotted NC membranes.

B. Phospholipase A2 (P3) (**Figure 88**)

1. HuScFv of clones P3/2 and P3/5 could not react with any protein band on the blotted NC membrane.

2. HuScFv of clones P3/6, P3/7, and P3/18 reacted with protein bands at *Mr* > 195, and ~45 kDa.

C. Natrin (P4) (**Figure 88**)

Both HuScFv of clones P4/8 and P4/21 reacted with protein bands at *Mr* > 195, and ~45 kDa.

D. Phospholipase A2 (P5) (**Figure 89**)

1. HuScFv of clones P5/1, P5/9, and P5/11 reacted weakly with protein bands at *Mr* > 195 and ~45 kDa.

2. HuScFv of clones P5/2, P5/3, P5/4, P5/5, P5/6, P5/7, and P5/10 11 reacted with protein bands at *Mr* > 195, and ~45 kDa.

3. HuScFv of clones P5/3, P5/4, P5/5, P5/6, and P5/7 could react with protein band at *Mr* ~20 kDa.

E. Neurotoxin (P8) (Figure 90)

1. HuScFv of clones P8/0/1, P8/9/1, P8/19/1 and P8/31/3 reacted with protein bands at *Mr* >195, ~45, and ~20 kDa.

2. HuScFv of clones P8/7/2 and P8/22/3 reacted with protein bands at *Mr* >195, and ~45 kDa.

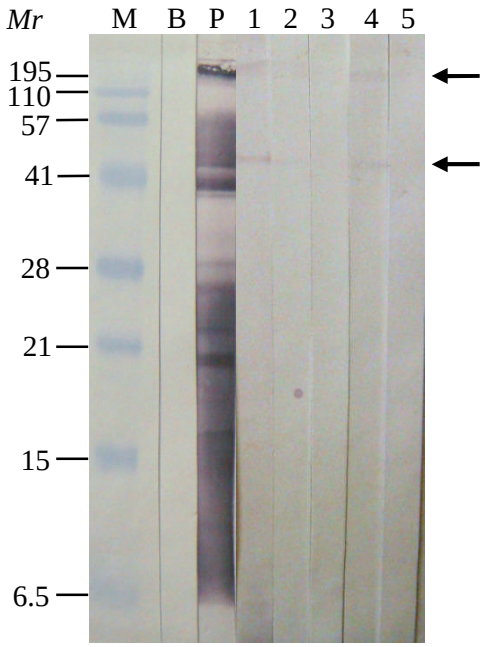

Figure 87 Western blot analysis of HuScFv prepared from HB2151 *E. coli* clones derived from bio-panning with **cobra venom factor** (P1).

Lane M, pre-stained protein standard marker

Lane B, background control (the crude Thai cobra venom reacted with anti-E-Tag MAb, goat anti-mouse immunoglobulin-alkaline phosphatase conjugate, and phosphatase substrate)

Lane P, positive control (the crude Thai cobra venom reacted with horse anti-Thai cobra venom, goat anti-horse immunoglobulin-alkaline phosphatase conjugate, and phosphatase substrate).

Lanes 1 to 5, test strips (incubated with whole cell lysates of HB2151 *E. coli* clones P1/1, P1/2, P1/3, P1/5, and P1/9, respectively.

Arrows indicate proteins at relative molecular masses >195, and ~45 kDa.

Numbers at the left of the figure are relative molecular masses (*Mr*).

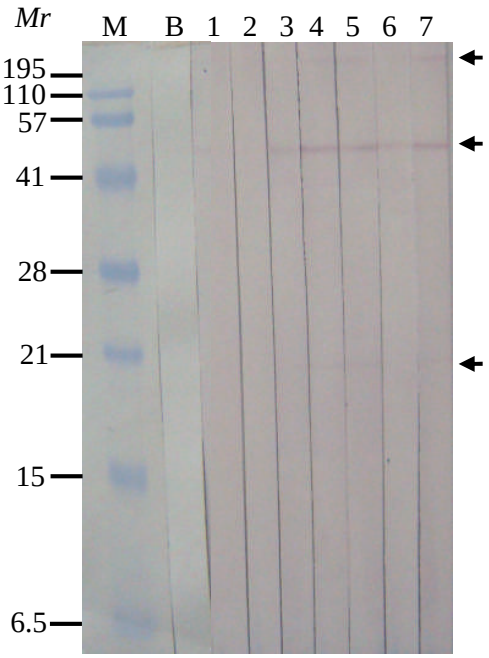

Figure 88 Western blot analysis of HuScFv prepared from HB2151 *E. coli* clones derived from bio-panning with **phospholipases A2** (P3) and **natrin** (P4).

Lane M, the pre-stained protein standard marker.

Lane B, background control (the crude Thai cobra venom reacted with anti-E-Tag MAb, goat anti-mouse immunoglobulin-alkaline phosphatase conjugate, and phosphatase sustrate.

Lanes 1 to 5, test strips (incubated with whole cell lysates of HB2151 *E. coli* clones P3/2, P3/5, P3/6, P3/7, and P3/18, respectively).

Lanes 6 to 7, test strips (incubated with whole cell lysates of HB2151 *E. coli* clones P4/8 and P4/21, respectively).

Arrows indicate proteins at relative molecular masses >195, ~45, and ~20 kDa.

Numbers at the left of the figure are relative molecular masses (*Mr*).

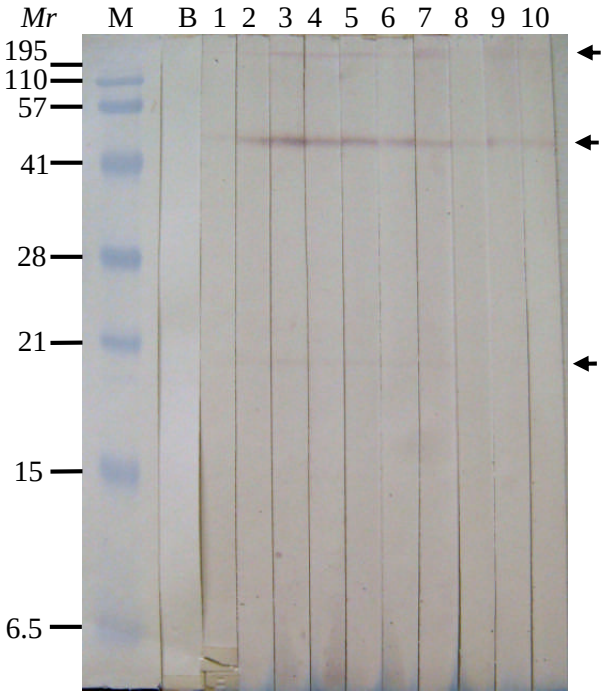

Figure 89 Western blot analysis of soluble HuScFv prepared from HB2151 *E. coli* clones that were derived from bio-panning with **phospholipases A2** (P5).

Lane M, the pre-stained protein standard marker.

Lane B, background control (the crude Thai cobra venom reacted with anti-E-Tag MAb, goat anti-mouse immunoglobulin-alkaline phosphatase conjugate, and phosphatase substrate).

Lanes 1 to 10, test strips (incubated with whole cell lysates of HB2151 *E. coli* clones P5/1, P5/2, P5/3, P5/4, P5/5, P5/6, P5/7, P5/9, P5/10, and P5/11, respectively).

Arrows indicate proteins at relative molecular masses >195, ~45, and ~20 kDa.

Numbers at the left of the figure are relative molecular masses (*Mr*).

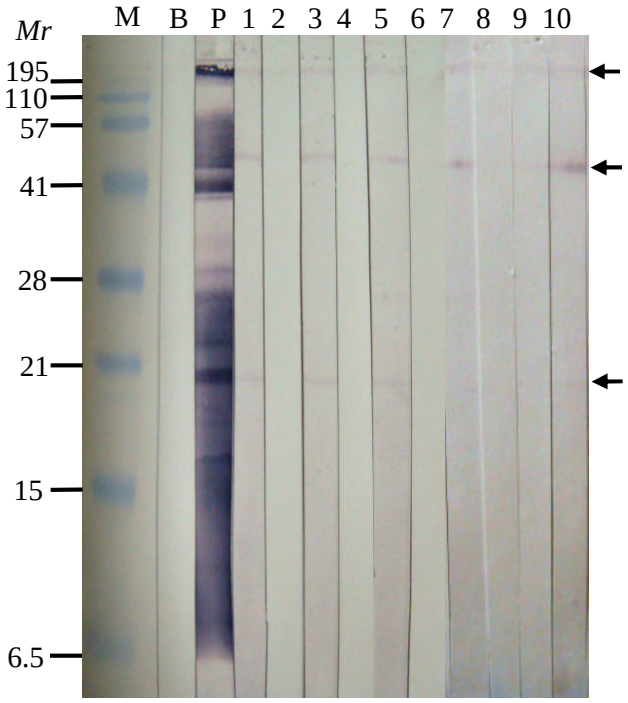

Figure 90 Western blot analysis of HuScFv prepared from *E. coli* HB2151 clones that were derived from bio-panning with **neurotoxin** (P8).

Lane M, the pre-stained protein standard marker

Lane B, background

Lane P, positive control

Lanes 1, 3, 5, and 7 to 10, test strips (the crude cobra venom incubated with **whole cell lysates** of HB2151 *E. coli* clones P8/0/1, P8/9/1, P8/19/1, P8/7/2, P8/10/2, P8/22/3 and P8/31/3, respectively).

Lanes 2, 4, and 6, test strip (the SDS-PAGE-separated crude cobra venom incubated with **periplasmic proteins** of HB2151 *E. coli* clones P8/0/1, P8/9/1 and P8/19/1, respectively). No HuScFv reactive band was found. The results indicate that the HuScFv were not secreted into the bacterial periplasm.

Arrows indicate proteins at relative molecular masses >195, ~45, and ~20 kDa.

Numbers at the left of the figure are relative molecular masses (*Mr*).

12. Production and purification of HuScFv

12.1 Fraction profile

Whole cell lysate prepared in TES buffer as described in **Section 10.1** of **Chapter IV** was subjected to HuScFv purification using affinity anti-E-Tag column (described in **Section 10.2** of **Chapter IV**).

To get high binding capacity of anti-E-Tag with HuScFv, pH of whole cell lysate was adjusted to 7.5 and protein sample loading was re-cycled 10 times (multiple passages). **Figure 91** shows the optical densities of proteins in the column flow through fluid (fractions numbered 1-10) and column-bound proteins (fractions numbered 11-25).

12.2 Detection of HuScFv

All protein fractions were monitored for the presence of HuScFv by dot-ELISA and Western blot analysis using anti-E-Tag MAb as a detection reagent. HuScFv in whole cell lysate before purification was used as positive control in dot-ELISA and WB (block S of **Figure 92** and lane S of **Figure 93**, respectively). Small amount of HuScFv was detected in the flow through fractions as shown in dot-ELISA and WB (block F of **Figure 92** and lane F of **Figure 93**, respectively).

HuScFv in the 1 M glycine, pH 3.0-eluted fractions numbered 11-25 were detected by dot-ELISA. Eluted fractions numbered 14-21 contained HuScFv (**Figure 92**, blocks 14-21) were pooled and analysed for molecular mass and purity by SDS-PAGE and WB. High purity of HuScFv were obtained and the molecular masses were ~25-27 kDa (lanes E in **Figures 93A** and **93B**).

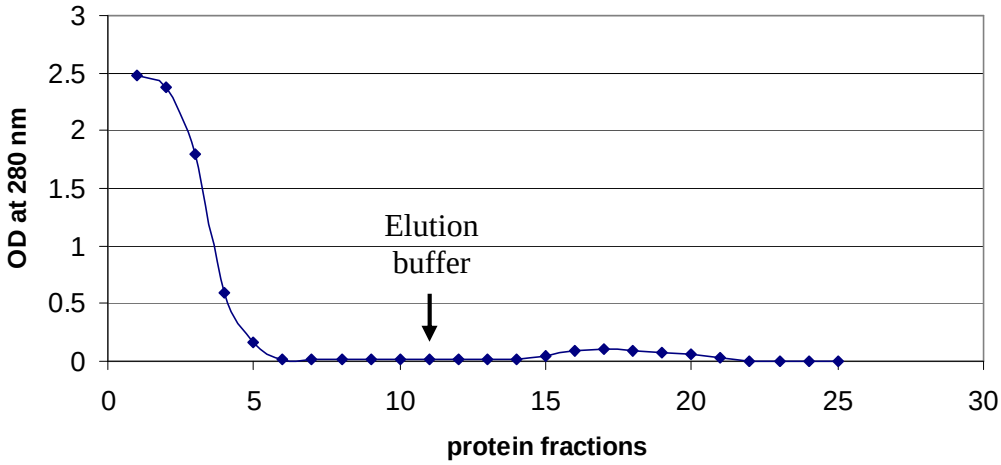

Figure 91 Optical densities of the proteins of *huscFv*-transformed *E. coli* lysate after passing through the affinity anti-E-Tag column

The unbound proteins (fractions numbered 1-10) from the affinity-anti-E-Tag column. Fractions numbered 11-25 are 0.1 M glycine, pH 3.0-eluted fractions.

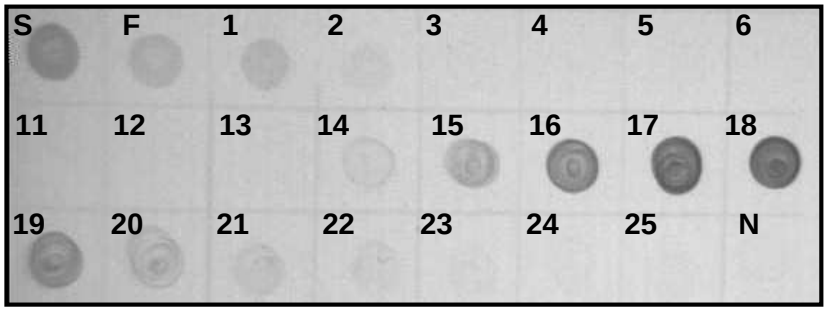

Figure 92 Dot-ELISA for detecting HuScFv in whole cell lysates of HB2151 *E. coli* and protein fractions after purification.

S, whole cell lysate of HB2151 *E. coli* containing *huscFv*-pCANTAB5E vector (positive HuScFv control)

F, and 1-6, column flow through fractions

11-25, fractions eluted with 1 M glycine, pH 3.0

N, negative control (whole cell lysate of normal HB2151 *E. coli*)

Fractions no. 14-21 contained HuScFv

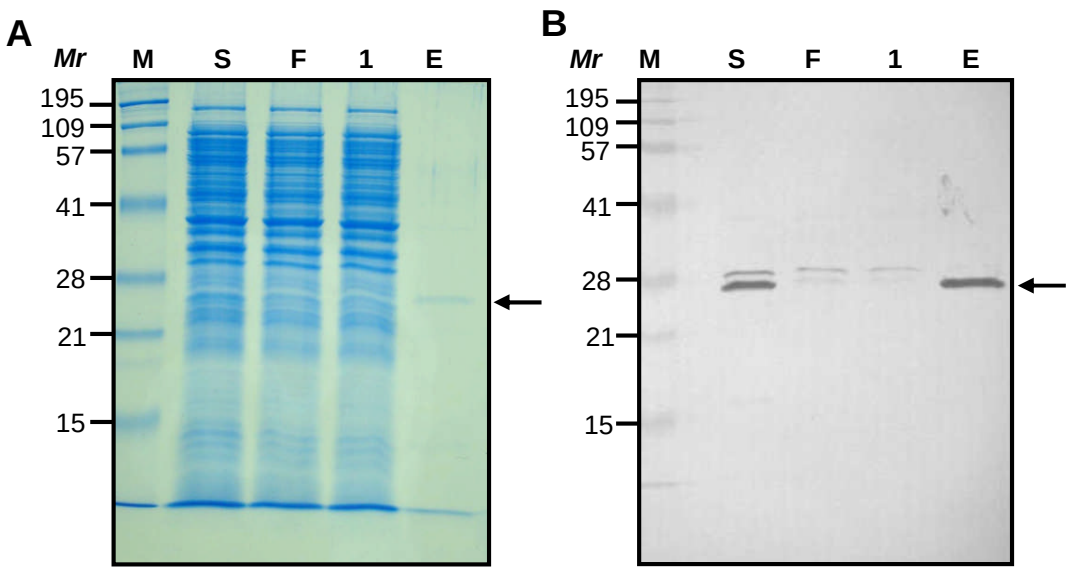

Figure 93 SDS-PAGE (**A**) and Western blot analysis (**B**) of proteins in various fractions from the affinity anti-E-Tag column

Lanes M, protein standard marker

Lanes S, whole cell lysate proteins of the HB2151 *E. coli* containing *huscFv*-pCANTAB5E

Lanes F, whole cell lysate proteins after passing through the anti-E-Tag column

Lanes 1, proteins in whole cell lysate of the transformed HB2151 *E. coli* in flow through fraction 1

Lanes E, proteins in glycine buffer eluted fractions (pool of fractions 14-21 of **Figure 92**)

13. *Mva*I RFLP of *huscFv* derived from bio-panning with P8 (predominantly contained long neurotoxin)

Figure 94 shows the banding patterns of the *Mva*I cut-*huscFv* sequences derived fron bio-panning with P8.

14. Mimotope searching

Epitopes of the purified HuScFv prepared from HB2151 *E. coli* clones P8/0/1, P8/9/1, P8/19/1, P8/7/2, P8/10/2, P8/22/3, and P8/31/3 were screened by bio-panning with the Ph.D.-12™ Phage Display Peptide Library Kit (NEW ENGLAND BioLabs, MA, USA) as described in section 11.1 of chapter IV. Three rounds of bio-panning were performed. **Figure 95** shows appearance of blue plaques of phages derived from the third round of bio-panning on an LB agar plate containing IPTG and X-gal. For HuScFv clones, *i.e.*, P8/0/1, P8/9/1, P8/19/1, P8/7/2, P8/10/2, P8/22/3 and P8/31/3, ten plaques were ramdomly screened for mimotope-encoding DNA sequences. After phage amplification, genomic DNA of phages were extracted and analyzed on agarose gel (**Figure 96**). Ten genomic DNA samples of clones P8/0/1, P8/7/2, P8/10/2 and P8/22/3 could be sequenced; nine genomic DNA samples of clones P8/19/1 and P8/31/3, and two genomic DNA samples of clone P8/9/1 could be sequenced. The sixty genomic DNA samples from total 70 genomic DNA samples of M13 phage contained the same mimotope-encoding DNA sequence as shown in **Figure 97**. The DNA sequence, 5'-CGTATTCACCGTACTCATAAACCACTCC ACCGGATT-3' was translated at (-1) frame by using TRANSEQ programe and it was found that it encoded for peptide: "**NPVEWFMSTVNT**".

This peptide sequence was aligned with long neurotoxin (1CTX α-cobratoxin of *N. naja* and *N. naja siamensis*, accession no. gi|229777|pdb|1CTX|) by using CLUSTALW 2.0.5 multiple sequence alignment. The peptide sequence, TVNT of mimotope was mostly matched with TVKT of long neurotoxin (**Figure 98**). The asparagine; "N" in the mimotpe is in the same group as the "lysine; K" of the long neurotoxin; thus both amino acids exert similar biological activity. The location of TVKT on three dimension structure (3DS) of the long neurotoxin was identified by using Cn3D 4.1 as shown in **Figure 99**.

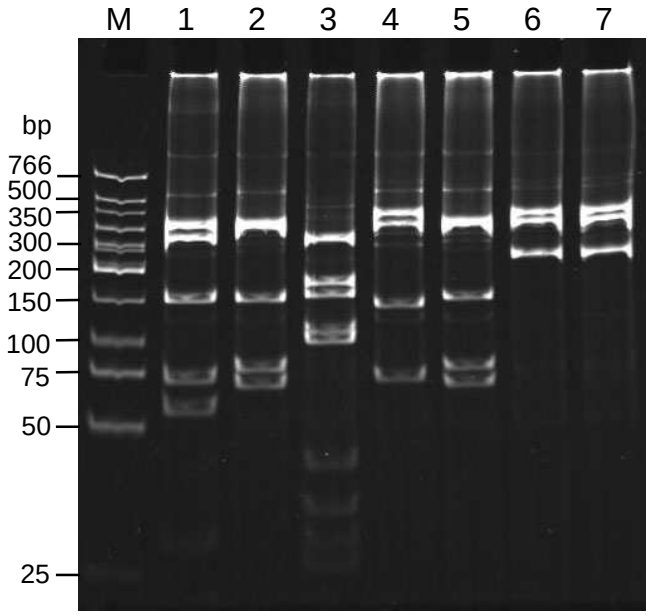

Figure 94 Patterns of *Mva*I RFLP of *huscFv* sequences

M, Low molecular size DNA ladder

*Mva*I-cut-*huscFv* sequences derived from 7 neurotoxin-binding transformed HB2151 *E. coli* clones which were analyzed on 12% acrylamide gel prepared in 1X TBE buffer and stained by using ethidium bromide

Lane 1, P8/0/1
Lane 2, P8/9/1
Lane 3, P8/19/1
Lane 4, P8/7/2
Lane 5, P8/10/2
Lane 6, P8/22/3
Lane 7, P8/31/3

Numers at the left of figure are DNA size in bp

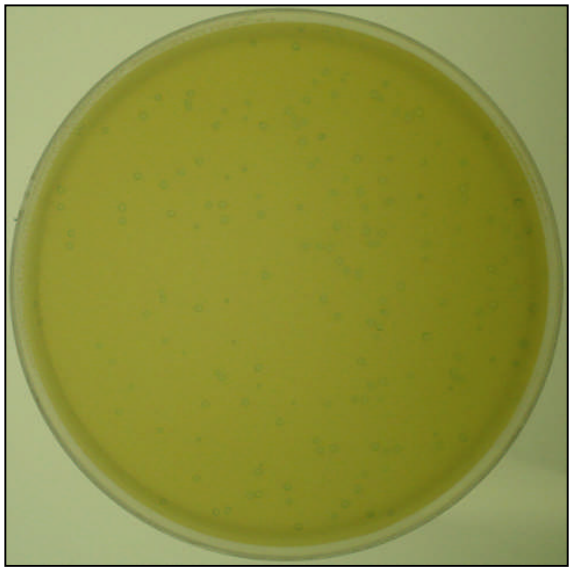

Figure 95 Appearance of plaques on an LB agar plate containing IPTG and X-gal

After the third round of bio-panning, the HuScFv-bound M13 phages were eluted and diluted to 10^{-4}.

Ten μl of the diluted phage solution were used to infect *E. coli* host cells. In order to amplify individual phage clones, the blue plaques were randomly picked and inoculated into LB medium containing appropriate *E. coli* host cells.

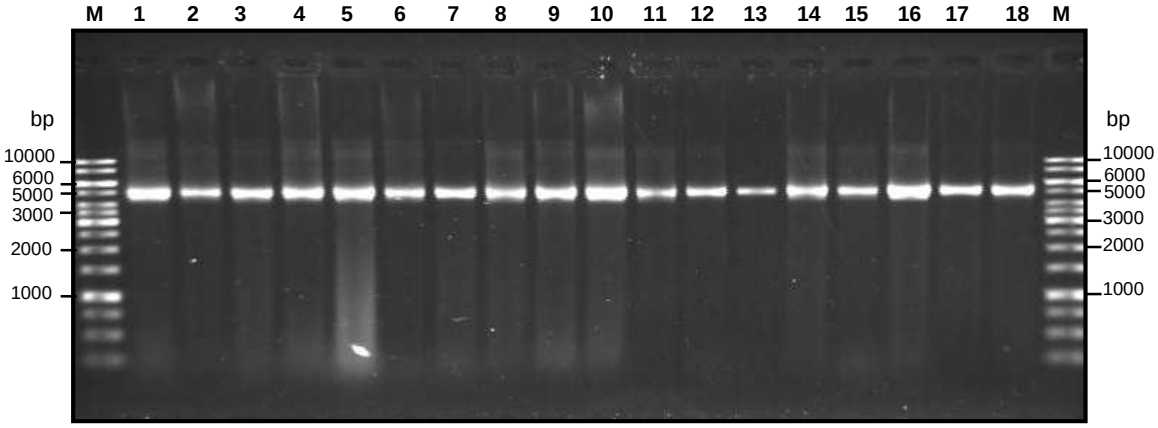

Figure 96 Eighteen representatives of M13 phage genome (Lanes 1-18) extracted from the amplified phage particles in LB medium by using phenol/chloroform and ethanol precipitation. Each M13 phage genome was analyzed on 0.8% agarose gel.

Lanes M, 1 kb DNA ladder marker

Lanes 1-18, the 18 representative genomic DNA samples of M13 phage derived from bio-panning with HuScFv

Numbers at both left and right are DNA sizes in bp

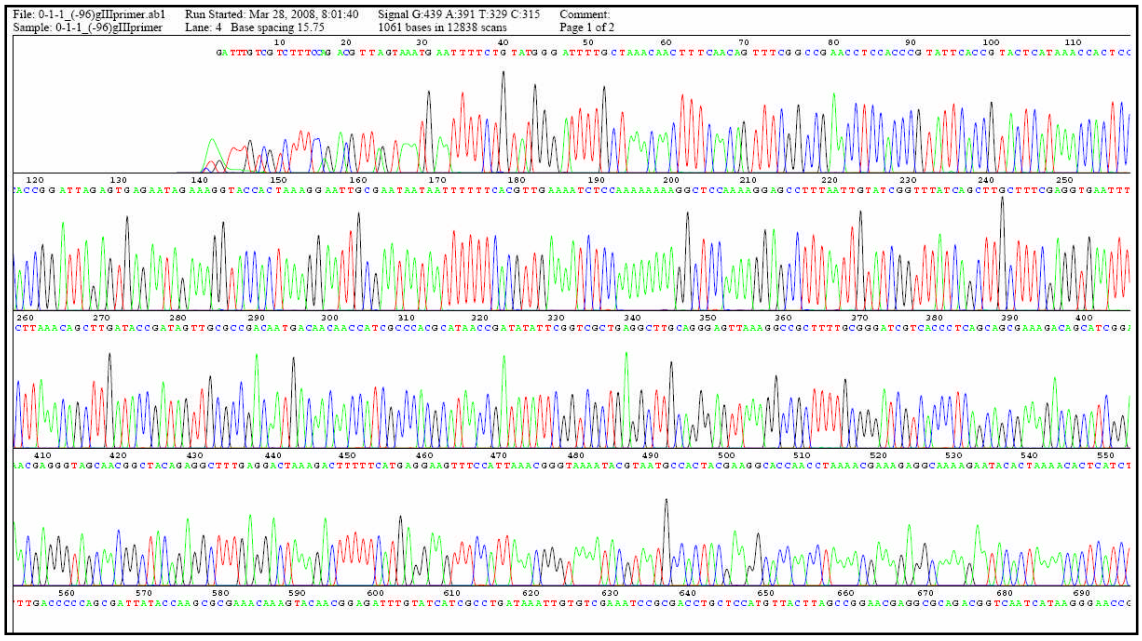

Figure 97 The representative DNA sequencing result of M13 phage genome selected from the third round of bio-panning with HuScFv that was prepared from HB2151 *E. coli* clone P8/0/1 plaque number 1.

The underlined DNA sequence is location of 12-mer peptides;

5’-CGTATTCACCGTACTCATAAACCACTCCACCGGATT-3’.

Translation at (-1) frame of this DNA sequence was performed by using TRANSEQ programe. This nucleotide sequence encodes for “**NPVEWFMSTVNT**”.

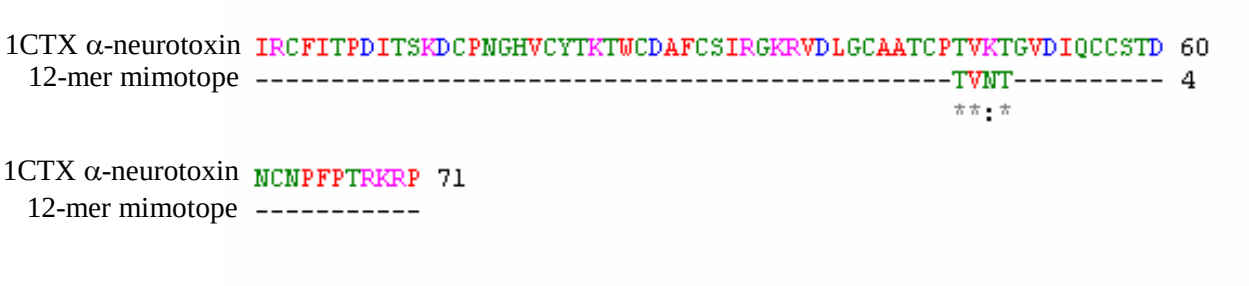

Figure 98 Pairwise alignment of the 12-mer mimotope "NPVEWFMSTVNT" and long neurotoxin *of N. naja siamensis* (α-neurotoxin) by using CLUSTALW 2.0.5 multiple sequence alignment.

" ***** " indicates the identical amino acid residues

" **:** " indicates the conserved substitution of amino acid residue

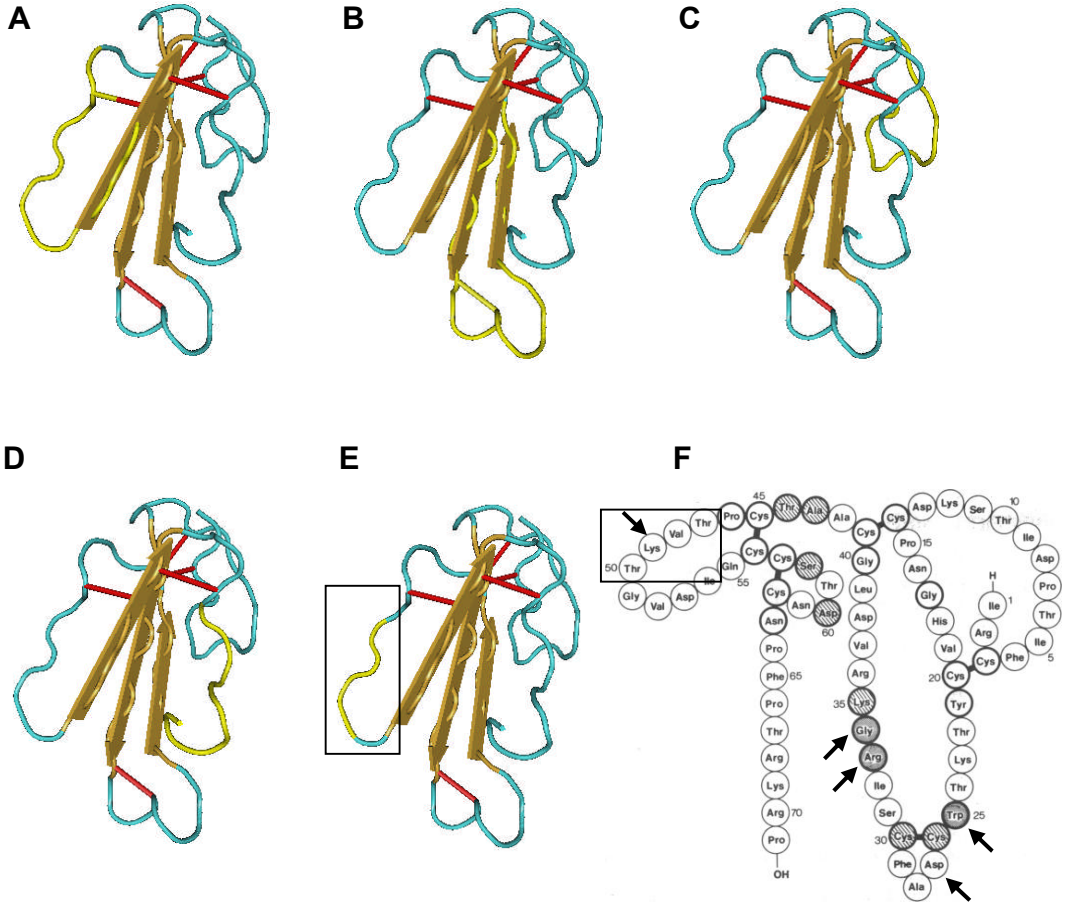

Figure 99 Three dimensional structure of long neurotoxin of *N. naja siamensis* (gi|229777|pdb|1CTX).

The beta sheet structure of protein is colored as dark yellow and blue color are long tube structure. The red colored lines indicate disulfide bonds.

A, Yellow ribbon indicates the small loop (amino acids 44-55)

B, Yellow ribbon indicates the large loop (amino acids 21-40)

C, Yellow ribbon indicates the small loop (amino acids 4-13)

D, Yellow ribbon indicates the tail of protein (amino acids 63-71)

E, Yellow ribbon indicates the TVKT on small loop of neurotoxin (amino acids 47-50)

F, Secondary structure of long neurotoxin collected from *N. naja*. Four amino acids TVKT in the box are homologous to the mimotopes of HuScFv identified in this study: "NPVEWFMS**TVN(K)T**"

Arrows indicate amino acid residues which play important role in **acetylcholine receptor binding** (Karlsson, 1979).

15. *In vivo* venom neutralization test

Purified HuScFv derived from individual phage clones were tested for their venom neutralizing activity *in vivo* according to WHO protocol**:** Venom (1.5 LD50) in 200 µl of NSS was mixed with purified HuScFv at 10x 1.5 LD50 derived from clones P8/0/1, P8/9/1, P8/19/1, P8/7/2, P8/10/2, P8/22/3 and P8/31/3 before injected intra-peritoneally into each mouse. Numbers of alive or dead mice were counted every 20 minutes until all of the mice that received only the venom (1.5 LD50) were dead (**Table 15**). HuScFv of clone 8/22/3 could rescue 100% of envenomed mice. HuScFv of clones P8/0/1, P8/9/1, P8/19/1, P8/7/2, and P8/10/2 were as effective as horse anti-venom and could rescue 83% of the envenomed mice. HuScFv of clone P8/31/3 could rescue 67% of the envenomed mice.

The amount of purified HuScFv at 50x 1.5 LD50 was used instead of 10x 1.5 LD50 to mix with the 1.5x LD50 of the venom and the mixture was injected into each mouse (except the venom control group). Then, five doses of purified HuScFv at 10x 1.5 LD50 were injected intra-peritoneally every ten minutes into each mouse of the groups that received HuScFv+venom. Mice of the group that received horse anti-venom were similarly treated with the HuScFv. Numbers of alive or dead mice were counted every 20 minutes. After all mice received only venom (1.5 LD50) had died, mice of the other groups were observed for one more hour (**Table 16**). HuScFv of clone P8/0/1, P8/19/1, P8/7/2, and P8/31/3 could rescue 100% of the envenomed mice as effective as horse anti-venom. HuScFv of clones P8/22/3, P8/10/2, and 8/9/1 could rescue 83%, 67%, and 50% of the envenomed mice, respectively.

Table 15 Survival rates of different groups of mice after receiving crude *N. kaouthia* venom pre-incubated with horse anti-venom or HuScFv from different phage clones compared to the group that received only venom.

Time (min)	Group																									
	1 Venom (1.5 LD50)			2 Horse anti-venom + venom			3 clone no. 8/0/1 + venom			4 clone no. 8/9/1 + venom			5 clone no. 8/19/1 + venom			6 clone no. 8/7/2 + venom			7 clone no. 8/10/2 + venom			8 clone no. 8/22/3 + venom			9 clone no. 8/31/3 + venom		
	A	D	%S	A	D	%S	A	D	%S	A	D	%S	A	D	%S	A	D	%S	A	D	%S	A	D	%S	A	D	%S
20	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**
40	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**
60	4	2	**67**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**
80	0	6	**0**	5	1	**83**	5	1	**83**	5	1	**83**	5	1	**83**	5	1	**83**	5	1	**83**	6	0	**100**	4	2	**67**

A, alive

D, dead

%S, percent survival rate

The protection test was performed according to the World Health Organization (WHO) protocol. The venom was pre-incubated with the antibody preparation at 37°C for 30 minutes before the mixture was intra-peritoneally injected into each mouse.

Table 16 Survival rates of different groups of mice after receiving crude *N. kaouthia* venom only or the venom pre-incubated with horse anti-venom or HuScFv from different phage clones. The horse anti-venom or HuScFv was then injected intraperitoneally every 10 minutes into each mouse of the groups that had received the venom+horse anti-venom or venom+HuScFv mixture. Five doses of the antibody were given after the initial injection. The experiments were terminated one hour after all the mice of the first group had been dead.

Time (min)	Group																									
	1 Venom (1.5 LD50			2 Horse anti-venom + venom			3 clone no. 8/0/1 + venom			4 clone no. 8/9/1 + venom			5 clone no. 8/19/1 + venom			6 clone no. 8/7/2 + venom			7 clone no. 8/10/2 + venom			8 clone no. 8/22/3 + venom			9 clone no. 8/31/3 + venom		
	A	D	%S	A	D	%S	A	D	%S	A	D	%S	A	D	%S	A	D	%S	A	D	%S	A	D	%S	A	D	%S
20	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**
40	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**
60	5	1	**83**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**
80	1	5	**17**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**
100	0	6	**0**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**
120	0	6	**0**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**
140	0	6	**0**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	6	0	**100**	5	1	**83**	6	0	**100**	6	0	**100**
160	0	6	**0**	6	0	**100**	6	0	**100**	3	3	**50**	6	0	**100**	6	0	**100**	4	2	**67**	5	1	**83**	6	0	**100**

A, alive

D, dead

%S, percent survival rate