CHAPTER IV
MATERIALS AND METHODS

1. Animals

In this study, ICR mice were used in the venom neutralization test. They were obtained from the National Laboratory Animal Center, Mahidol University, Salaya Campus, Nakhon-Pathom, Thailand.

Animal experiments were approved by Ethical Committee of Faculty of Allied Health Sciences, Thammasat University, Rangsit Center, Pathumthani, Thailand **(Appendix A).**

Animal manipulations were humanly performed using the guideline of the National Research Council of Thailand (NRCT).

2. Construction of human antibody phage display library

2.1 Source of human immunoglobulin genes

Human immunoglobulin genes were from two different sources: 1) peripheral blood mononuclear cells (PBMC) of 50 young adult Thai volunteers; and 2) 10 buffy coat samples of Thai blood donors. This part of the work was approved by Ethical Committee of Faculty of Allied Health Sciences, Thammasat University, Thailand **(Appendix B).**

2.1.1 Peripheral bloods of young adult Thai volunteers

Twenty-five ml of venous blood samples were individually collected from 50 young adult Thais (aged 18-28 years old; 25 female and 25 male). All volunteers gave written informed consents. Each blood sample was taken by using a 21-gauge needle connected to a VACUTAINER® CPT™ Cell Preparation tube containing sodium citrate (Beckton Dickinson, USA).

2.1.2 Buffy coat samples of 10 Thai blood donors

Buffy coat samples were from donated whole bloods of the Thai Red Cross which the plasma, red blood cells, and platelets had been removed. Each sample was from 250-300 ml of the whole venous blood. Each buffy coat sample was kept in CPD triple blood bag.

2.2 Isolation of peripheral blood mononuclear cells (PBMC) from whole venous blood and buffy coat preparations

PBMC were isolated within four hours after the blood/buffy coat collection to minimize messenger RNA (mRNA) degradation. The blood in VACUTAINER® CPT™ Cell Preparation tube was gently mixed with sodium citrate and the tube was centrifuged at 1,500 x *g*, 25°C for 30 minutes. PBMC were collected from the layer between the plasma and the packed red blood cells. They were washed two times with phosphate buffered saline, pH 7.4 (PBS, **Appendix C**) by centrifugation as above. Viable nucleated cells were enumerated by using 0.02% trypan blue dye exclusion method and hematocytometer. The number of cells in each preparation was calculated:

$$\text{Cell concentration (cells/ml)} = \frac{\text{number of viable cells}}{4} \times 10^4 \times \text{dilution factor}$$

For isolation of PBMC from the buffy coat, each buffy coat sample was mixed gently with equal volume of PBS. Three parts of the mixture was then overlaid on one part of Ficoll-Paque (Ficoll MW 400,000 with density 1.077 + 0.001 g/ml, Amersham Biosciences, Sweden) and the preparation was centrifuged at 2,000 x *g*, 25°C for 25 minutes. The PBMC located between the layers of plasma and red blood cells, were collected, washed two times with PBS, and the number of cells was enumerated.

2.3 Total RNA extraction

PBMC preparation (10^7 cells) was mixed well with one ml of TRIZOL® reagent (Invitrogen, USA). The mixture was kept at 25°C for 5 minutes. Chloroform (200 µl) was added to the tube and mixed well by inverting the tube for 15-30 seconds. The mixture was kept at 25°C for 2-3 minutes then centrifuged at 12,000 x *g*, 4°C for 15 minutes. The supernatant was collected into a new sterile microcentrifuge tube, 500 µl of isopropanol were added, and the tube was kept at 25°C for 10 minutes. The preparation was centrifuged as above. The supernatant was discarded and the pellet was washed with 75% ethanol; then air dried. Fifty µl of RNase-free distilled water (DW) were added to dissolve the RNA pellet.

2.4 Purity and integrity of the prepared RNA

Optical density (OD) of the RNA was measured at absorbances 260 and 280 nm (A_{260nm} and A_{280nm}). The A_{260nm} : A_{280nm} OD ratio of the RNA was in the range of 1.5 to 2.0, implying good RNA quality (purity).

Integrity of the RNA was determined by using 1% agarose gel electrophoresis in 20 mM Tris, 10 mM acetic acid, and 0.5 mM EDTA buffer, pH 8.3 (0.5x TAE buffer) (**Appendix D**). The gel was stained with ethidium bromide (0.5 μg/ml ethidium bromide in 0.5x TAE buffer) and the ribosomal RNA bands, *i.e.*, 28 S and 18 S were observed under U-V light by using U-V Transilluminator (Gel-Doc 2000, Bio-Rad). The presence of 28 S and 18 S rRNA bands indicated the RNA integrity.

2.5 Synthesis of the first strand of complementary DNA (cDNA)

The first complementary single stranded DNA (cDNA) was synthesized from the total RNA by using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Canada). The oligo $(dT)_{18}$ primer bound with poly-A tail of the mRNA and the first cDNA was produced by using RevertAid™ H Minus M-MuLV Reverse Transcriptase, that was genetically modified to lack RNase-H activity for preventing mRNA decay during the cDNA synthesis. Total RNA (0.1-5.0 μg) was mixed with 0.5 μg of the oligo $(dT)_{18}$ primer. RNase-free DW was added to a final volume of 12 μl. After gently mixing, the tube containing the mixture was centrifuged at 250 x *g* for 3-5 seconds and it was kept at 70°C for 5 minutes, then chilled in an ice-bath. Four μl of a 5x reaction buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 20 mM $MgCl_2$, and 50 mM DTT), 1 μl of 10 mM dNTP mixture, and 1 μl of ribonuclease inhibitor (20 units/μl) were mixed together and added to the oligo $(dT)_{18}$-primed mRNA preparation, the preparation was incubated at 37°C for 5 minutes. One μl of the RevertAid™ H Minus M-MuLV Reverse Transcriptase (200 units/μl) was added to the preparation and the tube was kept at 42°C for 60 minutes. The enzymatic reaction was stopped by heating the preparation at 70°C for 10 minutes, chilled in an ice-bath, and the preparation was ready for DNA amplification by PCR using human β-actin gene sequences as standard PCR control.

2.6 Preparation of human β-actin gene sequences

The human β-actin gene sequences were amplified using the previously prepared cDNA and forward primer; 5'-ACCTGACTGACTACCTCA-3' and reverse primer; 5'-GACTCCATGCCCAGGA-3'. The oligonucleotide primers bound to the DNA at position 623 and 869. The size of human β-actin amplicon was ~246 base pairs (bp). The PCR mixture for amplification of human β-actin gene sequences consisted of:-

Ingredient	**Volume (μl)**	**Final concentration**
Sterile UDW	16.8	-
PCR buffer+ KCl, 10x	2.5	1x
$MgCl_2$, 25 mM	1.5	1.5 mM
dNTP mix, 2.5 mM (each)	2.0	0.2 mM
Forward primer, 20 μM (each)	0.5	0.4 μM
Reverse primer, 20 μM (each)	0.5	0.4 μM
DNA template (cDNA)	1.0	-
Taq DNA polymerase (5 units/μl)	0.2	1 unit
Total	25	

PCR condition of human β-actin-encoded DNA sequence amplification was: initial denaturation of the cDNA template at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 1 minute. The final extension was performed at 72°C for 10 minutes.

2.7 Amplification of genes encoding variable heavy (*VH*) and kappa (*Vκ*) chains of the human immunoglobulins by PCR and verification of the DNA amplicons by agarose gel electrophoresis

2.7.1 VH gene (*VH*) amplification

DNA sequences encoding variable regions of human immunoglobulin heavy chains (*VH*) were amplified by using degenerate and non-degenerate oligonucleotide primers. All of the forward primers could bind specifically to the immunoglobulin framework 1 (FW1) of the *VH* and the reverse primers could bind specifically to the DNA encoding the joining segments (*JH*) of human *VH* sequences (**Figure 25**). The forward and reverse primer sets were designed from the VBASE (www.vbase.mrc-cpe.cam.ac.uk, accessed on Mar, 2005). ***Sfi*I endonuclease restriction site was included at 5' ends of each of the 14 forward primers, *i.e., VH1a, VH1b, VH1c, VH1d, VH2a, VH2b, VH3a, VH3b, VH3c, VH4a, VH4b, VH5a, VH6a,* and *VH7a*; designated Pr1. A short DNA linker encoding three repeats of four glycines and one serine (Gly_4Ser) was added at 5' end of each of the three reverse primers, *i.e., JH1245, JH3,* and *JH6*; designated Pr2.**

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* Primers designed for *VH* amplification are subjects of patent right reserve.

The PCR mixture for *VH* amplification consisted of:-

Ingredient	**Volume (μl)**	**Final concentration**
Sterile UDW	16.8	-
PCR buffer+ KCl, 10 x	2.5	1x
$MgCl_2$, 25 mM	1.5	1.5 mM
dNTP mix, 2.5 mM (each)	2.0	0.2 mM
Forward primer, 20 μM (each)[1]	0.5	0.4 μM
Reverse primer, 20 μM (each)[2]	0.5	0.4 μM
DNA template (cDNA)	1.0	-
Taq DNA polymerase (5 units/μl)	0.2	1 unit
Total	25	

[1] *VH1a, VH1b, VH1c, VH1d, VH2a, VH2b, VH3a, VH3b, VH3c, VH4a, VH4b,* VH5a, *VH6a,* and *VH7a* (designated Pr1)

[2] *JH1245*, *JH3*, and *JH6* (designated Pr2)

PCR condition of *VH* amplification was: initial denaturation of the cDNA template at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 1 minute. The final extension was performed at 72°C for 10 minutes.

2.7.2 Vκ gene (*Vκ*) amplification

In human, 60% of light chains are kappa (Vκ) and 40% are lamda (Vλ). For antibody phage display library construction in this thesis, only Vκ-coding DNA sequences were used to generate *huscFv* sequences. DNA sequences encoding Vκ were amplified by using degenerate and non-degenerate forward primers that could bind specifically to framework 1 (FW1) of *Vκ* sequences. Degenerate and non-degenerate reverse primers that could bind specifically to the joining segments (*Jκ)* were used. **Figure 26** shows diagram of primer binding to the template. All primers were also designed from the VBASE with some modification. ***Not*I endonuclease**

restriction site was included at 5' end of each of the 13 forward primers: *Vκ1a, Vκ1b, Vκ1c, Vκ1d, Vκ2a, Vκ2b, Vκ3a, Vκ3b, Vκ3c, Vκ4a, Vκ5a, Vκ6a,* and *Vκ6b*; designated Pr3. A DNA linker encoding Gly_4Ser was added at 5' end of each of the two reverse primers, *i.e., Jκ1234* and *Jκ5*; designated Pr4. The PCR reaction mixture for *Vκ* amplification consisted of:

Ingredient	**Volume (μl)**	**Final concentration**
Sterile UDW	16.8	-
PCR buffer + KCl, 10x	2.5	1x
$MgCl_2$, 25 mM	1.5	1.5 mM
dNTP mix, 2.5 mM (each)	2.0	0.2 mM
Forward primers, 20 μM[1]	0.5	0.4 μM
Reverse primers, 20 μM[2]	0.5	0.4 μM
DNA template (cDNA)	1.0	-
Taq DNA polymerase (5 units/μl)	0.2	1 unit
Total	25	

[1] *Vκ1a, V1b, V1c, V1d, V2a, V2b, V3a, V3b, V3c, V4a, V5a, V6a,* and *V6b* (designated Pr3)

[2] *Jκ1234* and *Jκ5* (designated Pr4)

PCR condition for *Vκ* amplification was: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 1 minute. The final extension was performed at 72°C for 10 minutes.

[*] Primers designed for *Vκ* amplification are subjects of patent right reserve.

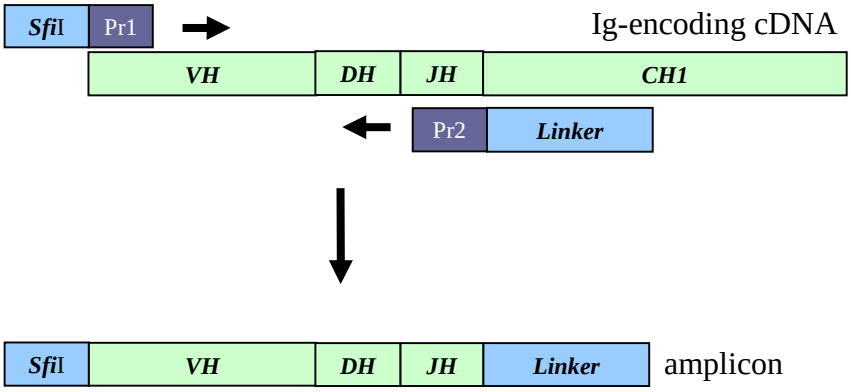

Figure 25 Diagram showing binding of forward (Pr1) and reverse (Pr2) primers to the immunoglobulin DNA template and positions of oligonucleotide linker and endonuclease restriction sites for PCR amplification of *VH* sequences.

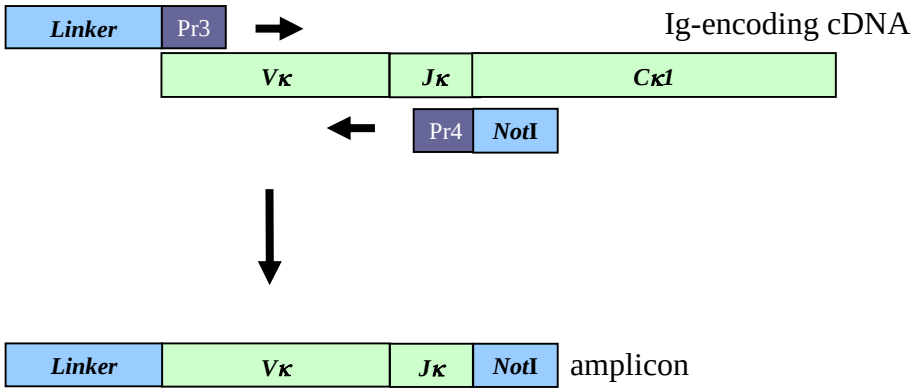

Figure 26 Diagram showing binding of forward (Pr3) and reverse (Pr4) primers to the immunoglobulin DNA template and positions of oligonucleotide linkers and endonuclease restriction sites for PCR amplification of *Vκ* sequences.

2.7.3 Agarose gel electrophoresis

Agarose electrophoresis for verification of the nucleic acid preparations was performed using 1% agarose gel (USB Corporation, USA) which was dissolved in 0.5x TAE buffer by heating in a microwave oven. The electrophoresis was performed using MINI-SUB® CELL CT apparatus (BIO-RAD). Molten agarose was casted and allowed to solidify and polymerized in a gel casting apparatus (MINI-SUB® CELL CT, BIO-RAD) at 25°C for 30-45 minutes. Sample for the electrophoresis was mixed with 10x loading buffer (0.4% bromophenol blue, 0.4% xylene cyanol and 50% glycerol) to yield the final concentration of 1x loading buffer. TAE buffer (0.5x) was used as an electrophoresis buffer. Electrophoresis was carried out at 100 Volts for about 45 minutes. The nucleic acid (DNA or RNA) was visualized after staining with ethidium bromide (0.5 μg/ml ethidium bromide in 0.5x TAE buffer) at 25°C for 10-15 minutes using U-V Transilluminator (Gel-Doc 2000, BIO-RAD).

The PCR amplicons of *VH* and *Vκ* in individual PCR mixture tubes were separately concentrated by ethanol precipitation. Equal volume of phenol/chloroform /isoamyl alcohol (25:24:1) was added into each tube of *VH*/*Vκ* PCR products and mixed. The preparation was centrifuged at 12,000 x *g*, 4°C for 5 minutes. The upper aqueous phase of each tube was collected into a new tube and equal volume of chloroform was added. After thorough mixing, the tube was centrifuged as above. The upper layer was collected into a new tube. Sodium acetate (3 M, pH 5.2, **Appendix D**) at 1/10 volume of the DNA preparation in each tube was added and mixed. Subsequently, two volumes of absolute ethanol were added and mixed with the content in each tube. The mixture was kept at -20°C for 1-2 hour(s), then it was centrifuged at 12,000 x *g*, 4°C for 20 minutes. After removing the supernatant, the pellet was washed once with 70% ethanol and dried. The DNA pellet was dissolved in a small volume of TE buffer (10 mM Tris, 1 mM EDTA buffer, pH 8.0, **Appendix D**). The DNA preparation was subjected to 1.5% agarose gel electrophoresis for verification of *VH* and *Vκ* sizes, *i.e.*, 400 bp and 350 bp, respectively and for purification of the DNA from the agarose gels. The *VH* and *Vκ* bands were separately excised from gels by using a razor blade and the excision was performed under the U-V light. The DNA was individually extracted from the gel pieces by using

GENECLEAN® II Kit (Q·BIO gene, USA). Each gel slice was weighed and the gel was placed into a 1.5-ml plastic tube. NaI solution (300 μl) was added to each 100 mg of the gel and incubated at 55°C for 1 minute in a heating block in order to melt the gel. Appropriate amount of well mixed-silica gel solution was added and mixed with the DNA preparation. The mixture was kept at 25°C for 5 minutes with occasional mixing. The DNA-bound silica gel was pelleted by centrifugation at 12,000 x *g*, 25°C for 15 seconds. The pellet was washed with the wash solution provided in the kit by centrifugation. The precipitate from the last wash was dried. The dried DNA was dissolved in sterile DW. The silica gel was removed by centrifugation. The amounts of purified *VH* and *Vκ* DNA were quantified by subjecting the prepared DNA in agarose gel eletrophoresis, ethidium bromide staining, and U-V transillumination. The intensity of each DNA band was compared with the intensity of the known amount DNA bands of the MassRuler™ DNA Ladder (Fermentas, Lithuania) run concurrently in the same gel. The pools of *VH* and *Vκ* genes were obtained in separate tubes.

2.8 Generation of human single chain DNA fragments encoding *VH*-linker-*Vκ* (*huscFv*) by splice overlapped extension-polymerase chain reaction (SOE-PCR)

2.8.1 Principle of SOE-PCR

Three sequential steps are essential for production of *huscFv* by SOE-PCR.

1. Binding between the two oligonucleotide linkers located at 3’ of *VH* and 5’ of *Vκ* sequences (**Figure 27A**).

2. Nucleotide addition by DNA polymerase to generate double stranded *huscFv* (**Figure 27B**).

3. Addition of two different outer primers at 5’ and 3’ end of individual *huscFv* strand (**Figure 27C**).

The PCR amplification of the complete *huscFv* sequences was then performed by the activity of DNA polymerase contained in the PCR mixture.

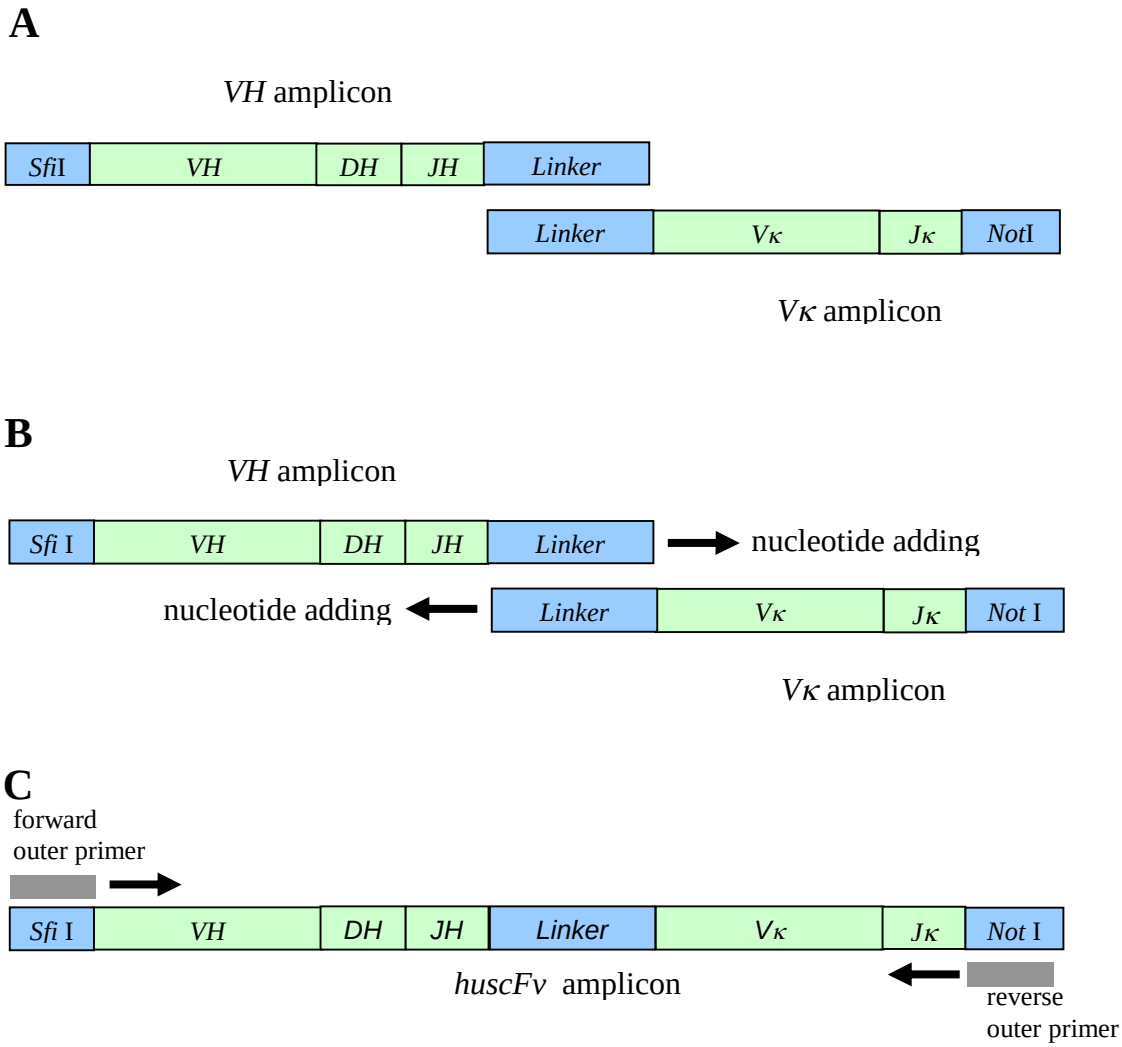

Figure 27 Sequential steps in SOE-PCR for constructing *huscFv* sequences.

2.8.2 SOE-PCR optimization

According to the principle outlined in **2.8.1**, there are factors that may influence the outcome of the SOE-PCR:

First, temperature might affect the ligation of the oligonucleotide linker to the *VH* and *Vκ* sequences and the binding of the outer primers to the *huscFv* template.

Second, addition of the outer primers either during or after *VH* and *Vκ* ligation *via* the linkers might yield different amplification results.

A. Influence of temperature

For optimization of the annealing of the two linkers (3'-end of *VH* and 5'-end of *Vκ*), the annealing temperatures were performed at 50°C, 55.5°C, 60.8°C and 66°C. The PCR mixture for annealing of the two nucleotide linkers was set as shown below.

Ingredient	**Volume (μl)**	**Final concentration**
Sterile UDW	16.8	-
PCR buffer + KCl, 10x	2.5	1x
$MgCl_2$, 25 mM	1.5	1.5 mM
dNTP, 2.5 mM (each)	2.0	0.2 mM
VH pool, 4 ng/μl	0.5	2 ng
Vκ pool, 4 ng/μl	0.5	2 ng
Taq DNA polymerase (5 units/μl)	0.2	1 unit
Total	25 μl	

The thermal cycles consisted of: initial *VH* and *Vκ* denaturation at 94°C for 5 minutes followed by 10 cycles of denaturation, annealing, and extension at 94°C for 1 minute, 50°C, 55.5°C, 60.8°C or 66°C for 1 minute, and 72°C for 1 minute, respectively. There was no final extension in this PCR.

After the two nucleotide linkers had annealed by the above reaction, forward and reverse outer primers (0.625 μM each) were added to the tube of the SOE-PCR mixture. The PCR was carried out by performing 20 cycles of denaturation, annealing, and extension at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes, respectively. The final extension was performed at 72°C for 5 minutes.

For optimization of the temperature for outer primer annealing to the *huscFv* sequences, the temperature for annealing of the two nucleotide linkers was set at 66°C. After the nucleotide linkers had been linked appropriately, four different temperatures were set for annealing of the outer primers, *i.e.*, 50°C, 55.5°C, 60.8°C, and 66°C. The PCR mixture was prepared:

Ingredient	**Volume (μl)**	**Final concentration**
Sterile UDW	16.8	-
PCR buffer + KCl, 10x	2.5	1x
$MgCl_2$, 25 mM	1.5	1.5 mM
dNTP, 2.5 mM (each)	2.0	0.2 mM
VH DNA pool, 4 ng/μl	0.5	2 ng
Vκ DNA pool, 4 ng/μl	0.5	2 ng
Taq DNA polymerase (5 units/μl)	0.2	1 unit
Total	25	

The thermal cycles for linking *VH* and *Vκ* together by the PCR were: initial denaturation of *VH* and *Vκ* sequences at 94°C for 5 minutes, followed by 10 cycles of denaturation, annealing, and extension at 94°C for 1 minute, 66°C for 1 minute, and 72°C for 1 minute, respectively. There was no final extension of this PCR.

The forward and reverse outer primers were added to yield 0.625 μM each. The thermal cycles for amplification of the *VH*-linker-*Vκ* sequences (*huscFv*) were: 20 cycles of denaturation, annealing, and extension at 94°C for 1 minute, 50°C,

55.5°C, 60.8°C or 66°C for 1 minute, and 72°C for 2 minutes, respectively. The final extension was performed at 72°C for 5 minutes.

B. Influence of outer primer adding step

For determining the suitability of the step to which the outer primers should be added to the PCR mixture for amplification of the complete *huscFv* sequences, the outer primers were added either during or after the *VH* and *Vκ* linking *via* the nucleotide linkers.

For outer primer adding **during** the *VH* and *Vκ* linking, the temperature for annealing of the two nucleotide linkers and the outer primers was set at 66°C. The PCR mixture was prepared:

Ingredient	**Volume (μl)**	**Final concentration**
Sterile UDW	15.8	-
PCR buffer + KCl, 10x	2.5	1x
$MgCl_2$, 25 mM	1.5	1.5 mM
dNTP, 2.5 mM (each)	2.0	0.2 mM
VH DNA pool, 4 ng/μl	0.5	2 ng
Vκ DNA pool, 4 ng/μl	0.5	2 ng
Taq DNA polymerase, 5 units/μl	0.2	1 unit
Forward outer primer, 20 μM	0.5	0.625 μM
Reverse outer primer, 20 μM	0.5	0.625 μM
Total	25	

The thermal cycles of this PCR mixture were: initial denaturation of *VH* and *Vκ* sequences was performed at 94°C for 5 minutes, followed by 30 cycles of denaturation, annealing, and extension at 94°C for 1 minute, 66°C for 1 minute, and 72°C for 2 minutes, respectively. The final extension was performed at 72°C for 5 minutes.

For outer primer adding **after** the *VH* and the *Vκ* linking, the temperature for annealing of the two nucleotide linkers and the outer primers was set at 66°C. The PCR mixture was prepared as shown below:

Ingredient	**Volume (μl)**	**Final concentration**
Sterile UDW	16.8	-
PCR buffer + KCl, 10x	2.5	1x
$MgCl_2$, 25 mM	1.5	1.5 mM
dNTP, 2.5 mM (each)	2.0	0.2 mM
VH DNA pool, 4 ng/μl	0.5	2 ng
Vκ DNA pool, 4 ng/μl	0.5	2 ng
Taq DNA polymerase, 5 units/μl	0.2	1 unit
Total	25	

The thermal cycles of this SOE-PCR mixture were: initial denaturation of *VH* and *Vκ* sequences at 94°C for 5 minutes, followed by 10 cycles of denaturation, annealing, and extension at 94°C for 1 minute, 66°C for 1 minute, and 72°C for 1 minute. There was no final extension at this step.

After the two nucleotide linkers had annealed by the above reaction, forward and reverse outer primers (0.625 μM each) were added to the tube of the SOE-PCR mixture. The PCR was carried out by performing 20 cycles of denaturation, annealing, and extension at 94°C for 1 minute, 66°C for 1 minute, and 72°C for 2 minutes, respectively. The final extension was performed at 72°C for 5 minutes.

After establishing the optimized SOE-PCR, high amount of *huscFv* sequences was produced by using the obtained pool of *VH* and *Vκ* sequences.

2.8.3 Preparation of *huscFv* sequences for ligation

After verifying the *huscFv* amplicon by subjecting the amplicon to agarose gel electrophoresis, ethidium bromide staining, and U-V light transillumination, the *huscFv* preparation was concentrated by using ethanol precipitation method as previously described in **Section 2.7.3.** The DNA pellet was dissolved in a small volume of sterile UDW.

The *huscFv* in the preparation was verified by 1.5% agarose gel electrophoresis. The expected band of the *huscFv* was 750 bp. The *huscFv* sequences were extracted from the gel by using GENECLEAN® II Kit (Q·BIO gene, USA). Amount of the recovered *huscFv* sequences was quantified by subjecting the prepared DNA to agarose gel eletrophoresis, ethidium bromide staining, and U-V transillumination. The intensity of the DNA band was compared with the intensity of the known amount DNA bands of the MassRuler™ DNA Ladder (Fermentas, Lithuania) run concurrently in the same gel. The *huscFv* sequences with good purity were obtained.

2.9 *Sfi*I restriction digestion of the *huscFv* sequences

The purified *huscFv* sequences (1 μg) were digested with 10 units of *Sfi*I (Fermentas, Lithuania) containing 1x concentration of buffer G (Fermentas, Lithuania) in a volume of 70 μl. After gently mixing and brief centrifugation, mineral oil (50 μl) was overlaid on top of the mixture. The enzymatic reaction was allowed to occur at 50°C for 4 hours in a water-bath. The *huscFv* sequences were precipitated from the enzymatic reaction mixture by using absolute ethanol as priously described in **Section 2.7.3.** The DNA pellet was dissolved in a small volume of sterile UDW. *Sfi*I-digested-*huscFv* sequences were obtained.

2.10 *Not*I restriction digestion of *huscFv* sequences

The *Sfi*I-digested-*huscFv* sequences (1 μg) were digested with *Not*I (10 units) (Fermentas, Lithuania). Enzymatic reaction mixture (70 μl) was set in a tube containing 1x concentration of buffer G (Fermentas, Lithuania). After gently mixing and brief centrifugation, mineral oil (50 μl) was overlaid on top of the mixture. The enzymatic reaction was allowed to occur at 37°C for 4 hours in a water bath. *Not*I and *Sfi*I-digested *huscFv* sequences were precipitated from enzymatic reaction mixture by

using absolute ethanol as described above. The amounts of *Not*I- and *Sfi*I-cut-*huscFv* sequences were quantified as described above in **Section 2.8**.

2.11 Ligation of the *Not*I- and *Sfi*I-cut- *huscFv* sequences into phagemid vector

The *Not*I- and *Sfi*I-cut-*huscFv* (150 ng) were ligated to *Sfi*I- and *Not*I- pre-cut phagemid vector (250 ng) (pCANTAB5E, Expression Module/Recombinant Phage Antibody System, GE Healthcare, UK) by using 10 units of T4 DNA ligase (New England BioLab® Inc., MA). Enzymatic reaction was set in a tube containing 1x concentration of OPA^+ buffer (Expression Module/Recombinant Phage Antibody System, GE Healthcare, UK) and 1 mM of ATP (Fermentas, Lithuania). For self-ligation control, pCANTAB5E (250 ng) phagemid vector was incubated with 10 units of T4 DNA ligase in the buffer as described above. After gently mixing and a brief centrifugation, both tubes of the ligation mixtures were kept at 16°C for 1 hour, and then at 4°C overnight (16 hours).

2.12 Preparation of recombinant *huscFv*-pCANTAB5E vectors for electroporation

Recombinant *huscFv*-pCANTAB5E phagemids were precipitated from the ligation mixture by using phenol/chloroform/isoamyl alcohol and ethanol precipitation as described in **Section 2.7.3** above. The dried DNA pellet was dissolved in a small volume of sterile UDW. The recombinant *huscFv*-pCANTAB5E phagemids were prepared.

2.13 Preparation of electrocompetent TG1 *E. coli* cells

Single colony of TG1 *E. coli* (Expression Module/Recombinant Phage Antibody System, GE Healthcare, UK) was picked from a minimum medium (MM) agar plate, inoculated into 2x YT broth (10 ml) (**Appendix E**), and incubated at 37°C with shaking at 250 rpm overnight. One ml of the culture was inoculated into 100 ml of fresh 2x YT broth and incubated at 37°C with shaking at 250 rpm until A_{600nm} = 0.5-0.7. The culture was chilled in an ice-bath for 30 minutes. The TG1 *E. coli* cells were pelleted by centrifugation at 3,500 x *g*, 4°C for 20 minutes. The cells were resuspended in 100 ml of ice-cold sterile 1 mM Hepes, pH 7.0. The preparation was centrifuged at 3,500 x *g*, 4°C for 20 minutes. TG1 *E. coli* cells were resuspended in

50 ml of ice-cold sterile 1 mM Hepes, pH 7.0, and then centrifuged as described above. The cells were gently resuspended with ice-cold sterile 1 mM Hepes, pH 7.0 (2 ml) and then centrifuged at 3,500 x *g*, 4°C for 20 minutes. The cells in the pellet were gently resuspended in sterile 10% glycerol (2 ml), and centrifuged at 3,500 x *g*, 4°C for 20 minutes. Finally, the cells were resuspended in 2 ml of sterile 10% glycerol and they were ready for electroporation (for reagents for electrocompetent cell preparation, please see **Appendix D**). The prepared competent cells were used within one hour after the preparation. The remaining competent cells in 50-μl aliquots were snap-frozen in liquid nitrogen and were kept at -70°C until use.

2.14 Transformation of the competent TG1 *E. coli* cells with the recombinant *huscFv*-pCANTAB 5E vector by electroporation

Fifty μl aliquot of the competent TG1 *E. coli* cells was slowly thawed in ice-bath, and transferred to a pre-chilled 0.2-cm electroporation cuvette (Eppendof, Germany). Two μl of the purified recombinant *huscFv*-pCANTAB5E suspension were added into the same cuvette and mixed gently. The cuvette was kept in an ice-bath for one minute. The electroporator (Eppendorf, Germany) was set at 25 μF, 2.5 kV, and 200Ω for 4.5-5.0 milliseconds. The outside wall of cuvette was blot-dried by using a tissue paper. The cuvette was placed into the electroporator. The electric pulse was generated by doubly clicking on the machine. After a beeb, one ml of 2x YT broth containing 2% glucose (2x YT-G) was immediately added into the cuvette and gently mixed by inverting the cuvette. The cell suspension was pipetted out from the cuvette and added into a new sterile 50-ml plastic tube. Nine ml of the 2x YT-G medium were added to mix with the content in the tube, one ml each at a time. The cell suspension (10 ml) was incubated at 37°C for 1 hour with shaking at 250 rpm and subsequently centrifuged at 3,500 x *g*, 25°C for 20 minutes. The cells were resuspended in one ml of 2x YT-G medium. Viable counts were performed by diluting the preparation in ten-fold dilutions: 10^{-2}, 10^{-4}, and 10^{-5}. Ten μl of the undiluted and diluted cell suspensions were individually plated onto selective agar plates containing 2% glucose and 100 μg/ml ampicillin, *i.e.*, SOBAG agar plate. The plates were incubated at 37°C overnight. Number of colonies of the transformed

E. coli were counted from the appropriate plate(s) and the number of transformed *E. coli* in the original undiluted suspension was calculated:

Colony forming units (cfu) = Number of colonies on plate x 100 x dilution factor

The remaining cell suspension was used in the phage rescuing by infecting the transformed cells with helper phages, *i.e.*, M13KO7 (Expression Module/Recombinant Phage Antibody System, GE Healthcare, UK).

2.15 Phage rescuing

The transformed TG1 *E. coli* cells were diluted with 2x YT medium to A_{600nm} of 0.3. Ampicillin and 2 M glucose were added to 100 μg/ml and 2% final concentration, respectively. The cell suspension was incubated at 37°C with shaking at 250 rpm for 1 hour. M13KO7 phages were added to the culture at a multiplicity of infection (moi) of 5:1*.

After adding M13KO7 phages, the culture was incubated at 37°C with shaking at 250 rpm for 1 hour. The culture was then centrifuged at 3,500 x *g*, 25°C for 10 minutes. The supernatant was carefully discarded. The cells in the pellet were gently resuspended with 10 ml of 2x YT medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin (2x YT-AK) without glucose. The culture was incubated at 37°C with shaking at 250 rpm overnight, centrifuged at 3,500 x *g*, 25°C for 20 minutes, and the supernatant containing recombinant phages was collected into a sterile 50-ml polypropylene centrifuge tube.

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* The number of pfu of M13KO7 phages for adding into the culture was determined as follows: (5×10^8 cells/OD at A_{600nm}) x (moi of 5) x (OD at A_{600nm} of ~0.5) x (final volume of cells).

2.16 Concentration of the recombinant phages and elimination of free HuScFv protein

The TG1 *E. coli* cells produce an amber suppressor tRNA which can read through (suppression) of the amber stop codon (cannot recognize the amber stop codon) located between the huscFv insert and gene 3 sequence of the pCANTAB5E. This suppression of the amber stop codon by TG1 *E. coli* is only ~20% efficiency; thus, the *E. coli* produce both soluble HuScFv with and without protein 3 (p3) and only the HuScFv with protein 3 can be displayed on phage surface. The presence of soluble HuScFv without protein 3 in the preparation may compete with the HuScFv-protein 3 molecules displaying on phages during subsequent use of the antibody phage library in bio-panning with the target antigen. Therefore, free HuScFv without protein 3 should be eliminated from the phage library. This could be done by polyethylene glycol (PEG, Sigma, USA) precipitation of the phage particles displaying HuScFv-protein 3, leaving the free HuScFv in the supernatant which could be removed by centrifugation. Briefly, 2 ml of PEG/NaCl were added to 10 ml of the recombinant phage-containing supernatant. The mixture was placed in an ice-bath for 60 minutes, centrifuged at 12,000 x *g*, 4°C for 20 minutes, and the supernatant was discarded. The remaining fluid in the tube was blotted away. The phage particles were resuspended appropriately with sterile PBS and filtered through a 0.45 μm membrane. Sterile glycerol was added into phage suspension to 50% final concentration and the phage suspension was stored at -20°C until use.

2.17 Determination of the phage titer

The titer of the phage preparation was determined as colony forming units (cfu) of the phage-infected *E. coli* cells which were ampicillin-resistant and could grow on the ampicillin-containing selective agar plate.

Single colony of TG1 *E. coli* was picked from minimum medium (MM) plate, inoculated into 2 ml of 2x YT medium, and incubated at 37°C with shaking at 250 rpm overnight. Fifty μl of the overnight culture were added into 5 ml of 2x YT medium (1% cell) and incubated at 37°C with shaking at 250 rpm until A_{600nm} reached 0.3-0.5 (log-phase). The phage solution was diluted at $1:10^7$, $1:10^8$, and $1:10^9$ in sterile PBS. Ten μl of each diluted phage solution were added into 100 μl of log-phase TG1 *E. coli*

preparation and kept at 25°C for 10 minutes. The phage-infected TG1 *E. coli* preparation was spreaded on 2x YT agar plate containing 2% glucose and 100 μg/ml ampicillin (2x YT-AG plate) and incubated at 37°C overnight. The numbers of transformed TG1 *E. coli* colonies was counted on the duplicate plates that gave not more than 200 and not less than 20 colonies. The phage titer of the phage library was calculated in terms of cfu per ml of phage solution:-

Colony forming unit (cfu)/ml = Number of colonies on plate x 100 x dilution factor

2.18 Titer of M13KO7 helper phages

The genome of M13KO7 helper phages contains several genes encoding both structural and non-structural proteins that are required for generation of complete progeny phage particles and also contains kanamycin-resistant gene. The titer of M13KO7 is the number of phages that can form bacterial plaques which are the area of the slow-growing bacterial cells that were infected with M13KO7 helper phages when comparing with the lawn of normal growing bacterial cells.

For titering the M13KO7 phages, single colony of TG1 *E. coli* grown on MM plate was inoculated into 2 ml of 2x YT broth and incubated at 37°C with shaking at 250 rpm overnight. Fifty μl of the overnight culture were added into 5 ml of fresh 2x YT medium (1% cells) and incubated at 37°C with shaking at 250 rpm until OD at A_{600nm} reached 0.3-0.5 (log-phase). The phage solution was diluted at $1:10^7$, $1:10^8$, and $1:10^9$ in sterile PBS. One hundred μl of each diluted phage solution were mixed into 100 μl of log-phase TG1 *E. coli* cells and incubated at 25°C for 10 minutes. The M13KO7-infected TG1 *E. coli* cells were added into 3 ml of molten top agarose which was pre-warmed at 45-47°C, mixed gently and each preparations was immediately poured on top of an LB agar plate. The plate was kept at 25°C for 5 minutes and then at 37°C overnight. The numbers of TG1 *E. coli* plaques on the duplicate plates were counted. The titer of M13KO7 plaques was calculated in term of plaque forming units per ml of the M13KO7 phage solution**:**

Plaque forming units (pfu)/ml = Number of plaques on plate x 10 x dilution factor

3. Characterization of human antibody phage display library

3.1 Amplification of *huscFv*

Twenty well-isolated colonies of TG1 *E. coli* transformants were randomly selected from a selective agar plate (SONAG) for *huscFv* amplification by PCR. In this PCR, *huscFv* inserts in the pCANTAB5E phagemids were amplified directly from bacterial colony by using a forward nucleotide primer designated pCANTAB5-*R1* (5'-CCATGATTACGCCAAGCTTTGGAGCC-3') that bound specifically to pCANTAB 5E vector closed to the 5' end of *Sfi*I restriction endonuclease site, and a reverse nucleotide primer designated pCANTAB5-*R2* primer (5'-GCTAGATTTCAAAAC-AGCAGAAAGG-3') that bound specifically to pCANTAB 5E vector closed to the 3' end of *Not*I restriction endonuclease site. The PCR mixture was prepared as shown below:

Ingredient	**Volume (μl)**	**Final concentration**
Sterile UDW	15.8	-
PCR buffer + KCl, 10x	2.5	1x
$MgCl_2$, 25 mM	1.5	1.5 mM
dNTP, 2.5 mM (each)	4.0	0.2 mM
pCANTAB-*R1*, 50 μM	0.5	0.4 μM
pCANTAB-*R2*, 50 μM	0.5	0.4 μM
Taq DNA polymerase, 5 units/μl	0.2	1 units
Total	25	

A sterile 10-μl pipette tip was used to pick a small portion of each of the 20 well-isolated single colonies on the selective agar plate and was individually resuspended directly in a PCR mixture. Small volume of each PCR mixture was streaked on replica agar plate (SOBAG plate or 2x YT-AG plate).

For the thermal cycles of *huscFv* amplification, the first denaturation was performed at 94°C for 10 minutes to allow bacterial cell lysis followed by 30 cycles of denaturation step at 94°C for 1 minute, annealing step at 55°C for 2 minutes, and

extension at 72°C for 2 minutes. The final extension was performed at 72°C for 2 minutes.

3.2 *Mva*I restriction fragment length polymorphism (*Mva*I RFLP) of *huscFv*

The experiment was performed for determining the diversity of the *huscFv* sequences. The restriction pattern of *huscFv* sequences from each *E. coli* clone was observed. The diversity was determined. Two μl of each of the PCR amplicon from 3.1 was cut by using 2 units of *Mva*I restriction endonuclease (Fermentas, Lithuania) in 1x buffer for *Mva*I in 10 μl final volume of enzyme digestion. The mixture was incubated at 37°C for 4 hours, and then the *Mva*I restriction endonuclease was inactivated by heating at 80°C for 20 minutes.

To analyze the pattern of *Mva*I-cut-*huscFv*, 12% polyacrylamide gel prepared in 89 mM Tris, 89 mM boric acid, and 2 mM EDTA buffer, pH 8.3 (1x TBE buffer) as described in **Appendix C** was prepared. The Low Molecular Weight DNA Ladder (New England BioLab® Inc., MA) or GeneRuler™ 100 bp DNA Ladder (Fermentas, Vilnius, Lithuania) was run parallel with *Mva*I-cut-*huscFv* on the same polyacrylamide gel slab by using electricity at 20 mA per gel. The DNA in polyacrylamide gel was visualized after staining with ethidium bromide (0.5 μg/ml ethidium bromide) by using a Transilluminator (Gel-Doc 2000, Bio-Rad).

4. Proteomics of *N. kaouthia* venom

Strict safety precautions and universal precautions were practiced when handling and storing the snake venom.

4.1 Preparation of venom for the two dimensional gel electrophoresis (2DE)-based-proteomic analysis

Crude *N. kaouthia* venom in lyophilized form was purchased from Queen Saovabha Memorial Institute (QSMI), Thai Red Cross, Bangkok, Thailand, after the authority's permission. The lyophilized venom was dissolved with small volume of sterile DW and the protein concentration was measured by using Bradford reagent (Bio-Rad, USA). The venom was centrifuged at 12,000 x *g*, at 4 °C, for 5 minutes to remove insoluble material. The supernatant was then precipitated by using the 2D Clean-Up kit (Amersham Biosciences, CA, USA). The precipitate was resuspended in

an appropriate volume of DeStreak™ Rehydration solution (Amersham Biosciences). Insoluble material was removed by centrifugation as above. The protein concentration was re-determined by using the 2D Quant kit (Amersham Biosciences).

4.2 Two dimensional gel electrophoresis (2DE)

For the first dimensional-electrophoresis, each IPG strip (Amersham Biosciences) was placed into the Ettan IPG Phor Electrofocusing System (Amersham Biosciences) and the IPG strip was allowed to rehydrate at 20 °C for 12 hours in the DeStreak™ Rehydration solution. Electrophoresis was performed at 300 V for 30 minutes, 1,000 V for 30 minutes and 5,000 V for 72 minutes. For the second dimension-electrophoresis, the focused IPG strip was equilibrated in a reduction buffer [50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue, and 1% (w/v) dithiothreitol], at 25 °C for 15 minutes. The strip was then equilibrated in an alkylation buffer [50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue, and 2.5% (w/v) iodoacetamide], at 25 °C for 15 minutes. SDS-PAGE was carried out in a 15% polyacrylamide gel cast in the Mini PROTEAN® 3 Cell (Bio-Rad), at 10 mAmp/gel during the first 15 minutes and at 20 mAmp/gel until the tracking dye reached the lower edge of the gel. Then the gel was stained by Coomassie Brilliant Blue R-250 dye for the proteomic study.

4.3 2DE-based-liquid chromatography/tandem mass spectrometry (2DE-LC/MS-MS)

The venom proteins in stained-2DE gel were carefully excised from the gel. The gel plugs were appropriately destained, and individual gel pieces were digested with trypsin according to the method of Kinter and Sherman (Kinter and Sherman, 2000). For the peptide analysis, the LC/MS-MS model Finnigan LTQ Linear Ion Trap Mass Spectrometer was used. The HPLC system was a Finnigan Surveyor™ MS pump with a flow splitter. The column size was 0.18 x 100 mm, *i.e.*, C_{18} (Thermo Electron, MA, USA). The flow rate was 200 µl/minutes. The two mobile phases were: A) water with 0.1% formic acid, and B) acetonitrile with 0.1% formic acid. After washing to remove unbound peptides with A, the following gradients of B were applied to the column: 2-60% B in 20 minutes, 65-80% B in 5 minutes, and 80-2% B in 2 minutes.

Peptides in the eluates were analyzed by mass spectrometer (Finnigan LTQ) which used NanoSpray, positive ion in ionization mode at a capillary temperature of 200 °C with a 1.8 kV spray needle. The scan sequence was full-scan MS and MS-MS scan with mass range 400-1,600 *m*/*z*. The acquisition modes were Normal, Data Dependent™ and Dynamic Exclusion™.

4.4 Two-dimensional liquid chromatography/tandem mass spectrometry (2D-LC/MS-MS)

N. kaouthia venom was digested with trypsin in a solution according to the protocol of Kinter and Sherman (Kinter and Sherman, 2000). In the analysis of the tryptic peptides by 2D-LC/MS-MS, the peptides were separated in strong cation exchange resin in a 0.32 x 100 mm microcapillary column (Thermo Electron). The loaded peptide mixture was eluted by using gradients of ammonium chloride (0, 20, 60, 80, 100, and 200 mM) into an ion trap column for desalting. Individual desalted- and fractionated-peptides were applied to HPLC (C_{18}) as described in **Section 4.3** for the 2DE-based-LC/MS-MS before peptide mass spectrum scanning.

4.5 Data analysis for 2D-LC/MS-MS

The ion spectra of peptides generated by mass spectrometry were interpreted by using the Turbo SEQUEST algorithm in the BioWorks™3.1SR1 software package (Thermo Electron) and nr.fasta database. Protein search parameter include a precursor peptide mass tolerance of ±1.25 amu, fragment mass tolerance of ±0.4 amu, methionine (M) oxidation, and threonine (T) or serine (S) phosphorylation. For tryptic status requirement, at least one end of the peptide must be a tryptic site. The identified peptides were further evaluated using charge state *versus* cross-correlation number (X_{corr}). The criteria for positive identification of peptides were X_{corr} >1.5 for singly charged ions, X_{corr} > 2.0 for doubly charged ions, and X_{corr} >2.5 or triply charged ions. A delta correlation (ΔC_n) > 0.08 was used as a cut-off for peptide acceptance. The minimum number of one peptide per protein was specified by software.

4.6 Matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF)

MALDI-TOF was used to generate mass spectrum from trypsin-digested peptides and was performed at the BSU, BIOTECH, NSTDA, Thailand. For the parameters used in homology search, database was the NCBInr 20070601 which contained 4,988,250 protein sequences (1,724,794,729 amino acid residues). The 802,011 protein sequences belonging to chordata (vertebrates and relatives). Type of search was peptide mass fingerprint (PMF) which was generated by trypsin digestion. The carbamidomethyl (C) amino acid modification was fixed and the variable amino acid modification was oxidation (M). The mass value was monoisotopic. The peptide mass tolerance was ± 0.8 Da. The peptide charge state was +1 and the maximum missed cleavage was one.

5. Fractionation of protein components of *Naja kaouthia* venom

5.1 Column preparation

Components of crude *N. kaouthia* venom were separated by using ion exchange column chromatography followed the method of Karlsson (1971) with modifications.

The column size was 1.3 x 44 cm. Weak cation exchanger matrix [Macro-Prep® CM support (Bio-Rad, CA, USA)], carboxylate group ($-COO^-$), pre-swollen and stored in 20% ethanol, was used. The CM support was equilibrated in 0.2 M ammonium acetate buffer, pH 6.5 by changing the buffer several times. The equilibrated CM support was kept at 25°C overnight before packing into a glass column. The 0.2 M ammonium acetate buffer, pH 6.5 was pumped into the column by using peristaltic pump at a flow rate 0.5 ml per minute until pH of the output buffer was equal to the pH of input buffer (0.2 M ammonium acetate buffer, pH 6.5).

5.2 Preparation of *N. kaouthia* venom for the column chromatography

Crude *N. kaouthia* venom in lyophilized form was purchased from Queen Saovabha Memorial Institute (QSMI), Thai Red Cross, Bangkok, Thailand. The lyophilized venom was dissolved with small volume of sterile DW and the protein concentration was measured by using Bradford reagent (Bio-Rad, USA). Crude venom of known concentration was then diluted in 0.09 M ammonium acetate, pH 6.5 (5 ml). The solution was centrifuged at 12,000 x *g*, 4°C for 10 minutes. The soluble

part of venom was collected and its temperature was brought to 25°C before loading into column.

5.3 Protein separation by ion exchange column chromatography

Ammonium acetate (0.09 M, pH 6.5) was pumped into the column to replace the 0.2 M ammonium acetate buffer, pH 6.5 using a flow rate of 0.5 ml per minute. Crude *N. kaouthia* venom dissolved in 0.09 M ammonium acetate, pH 6.5 (5 ml) was loaded into the column and unbound proteins were washed out by using 0.09 M ammonium acetate, pH 6.5. The column unbound proteins in the column flow through was collected in 3 ml-fractions. The proteins in unbound fractions were monitored by measuring A_{280nm}. When OD at A_{280nm} was equal to zero, the column bound proteins were eluted out by applying a linear gradient of 0.14 to 1.4 M ammonium acetate, pH 6.5. Three ml fractions were collected. The remaining proteins in the column were finally eluted by applying 1.4 M ammonium acetate, pH 6.5 until the OD at A_{280nm} was zero.

After monitoring the OD of proteins in each fraction at A_{280nm}, a graph was plotted: X axis for fraction number, and Y axis on left hand side for OD at A_{280nm} , and Y axis on right hand side for the concentrations of the ammonium acetate. The fractions of each protein peak from the graph were pooled. Each pool was concentrated by using Amicon® Ultra-15 Centrifugal Filter Device (Milli® Pore, MA, USA) with molecular weight cut-off at 5 kDa. The diluent was also changed from ammonium acetate buffer to DW. The protein concentration was measured by using Bradford reagent (Bio-Rad, USA) and bovine serum albumin (BSA) as protein standard. The protein in each peak was analyzed on 15% sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE) and staining with Coomassie™ Brilliant Blue G-250 dye.

6. Protein quantification by Bradford reagent

The microtiter plate protocol of Bio-Rad Protein Assay (Bio-Rad, USA) was used for protein quantification. One part of the Dye Reagent Concentrate was diluted with four parts of double, de-ionized distilled water and filtered through a Whatman no. 1 filter to remove insoluble particles. BSA was used as protein standard. Five dilutions of BSA were prepared at 0.05 to 0.5 mg/ml which was the linear range of

microtiter plate assay. Ten microliters of the diluted BSA and sample was pipetted into individual wells of the microtiter plate (Nunc-Immunotube™ Module, Denmark) in duplicate. Diluted dye reagent (200 μl) was added to each well and mixed. The mixture was kept at 25°C for 5 minutes. The absorbance of the colored content in each well was measured within 1 hour at A_{595nm}. The standard curve of BSA OD and concentrations was constructed.

7. Sodium dodecyle sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis (WB)

7.1 Sodium dodecyle sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE)

7.1.1 Protein separation

The Mini-PROTEAN-3 Cell System (Bio-Rad, USA) and the Laemmli buffer system were used for SDS-PAGE. In the Laemmli system, a discontinuous buffer system incorporates SDS in the buffer and protein is denatured by heating in buffer containing SDS and 2-mercaptoethanol (β-ME) which is a thiol reducing agent for reducing the disulfide bonds of polypeptides. The polypeptide was shaped as a rod and a uniform charge-to-mass ratio proportional to polypeptide molecular weight. Protein can be separated according to molecular weight (Laemmli, 1970).

The 4% stacking polyacrylamide gel was prepared in 0.5 M Tris-HCl, pH 6.8 and 12% or 15% resolving gel was prepared in 1.5 M Tris-HCl, pH 8.8 (**Appendix D**). The protein sample was diluted in either SDS reducing (with β-ME) or non-reducing buffer (without β-ME). Electricity was applied, *i.e.*, 10 mA per gel plate to drive all proteins through 4% stacking gel and then 20 mA of electricity was used per gel plate during protein separation in 12% or 15% resolving gel. The separated protein(s) in gel was/were stained with Coomassie Brilliant Blue R-250 or G-250 (USB corporation, Cleveland, OH, USA). The relative molecular masses (*Mr*) of protein bands were determined by comparing with pre-stained standard protein marker (Bio-Rad, USA) run concurrently in the same gel slab.

7.1.2 Staining of proteins in the polyacrylamide gel

A. Coomassie Brilliant Blue R-250 dye

The protein(s) in polyacrylamide gel was/were fixed and stained at the same time by using Coomassie Brilliant Blue R-250 dye [0.25% (w/v) Coomasie Brilliant Blue R-250, 45.4% methanol, and 9.2% glacial acetic acid in DW]. The gel was soaked with appropriate volume of the dye solution that covers the entire gel to prevent dye sedimentation on gel during soaking. The gel was kept in dye solution at 25°C for an hour or longer depends on the amount of protein(s) in the gel. After staining, the excess dye in the gel was de-stained by soaking the gel with high methanol de-staining solution (45.4% methanol, 7.5% glacial acetic acid, and 2.5% glycerol in DW) at 25°C for one hour or longer. The gel was then kept at 25°C overnight in low methanol de-staining solution (5% methanol, 7.5% glacial acetic acid, and 2.5% glycerol in DW) to generate more clear background on the gel. The gel was subsequently washed several times with 1% glycerol in DW to remove methanol and acetic acid and the protein bands were visualized or scanned by using a scanner machine or photographed. For long-term storage of the stained gel, the gel was dried on cellophane membrane and kept at 25°C.

B. Coomassie Brilliant Blue G-250 dye

The Coomassie Brilliant Blue G-250 can stain protein in nanogram scale. Containers and all equipment used for G-250 staining should be cleaned without protein or other substances that can be the source of non-specific or high background staining. Protein in polyacrylamide gel was fixed by using freshly prepared fixing buffer (1% *o*-phospholic acid and 20% methanol in UDW) at 25°C for an hour and then was incubated with freshly prepared dye solution [0.1% (w/v) Coomassie Brilliant Blue G-250, 8% ammonium sulfate, and 20% methanol in UDW] at 25°C overnight. The gel was removed from the dye solution and placed into a new container containing neutralizing buffer [1.2% (w/v) Tris-base in UDW, pH 6.5]. The gel was soaked in neutralizing buffer for 2-3 minutes and then washed with 25% ethanol before placing in a container containing stabilizing buffer [20% (w/v) ammonium sulfate in UDW] and kept at 25°C for 24 hours. The proteins in the gel

were visualized by either a scanner machine or photopgraphed. For long-term storage, the gel was dried on membrane and it could be kept at 25°C.

7.2 Western blot analysis (WB)

The protein(s) separated in the polyacrylamide gel was/were transferred onto NC membrane by using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, USA) (**Figure 28**). The protein-containing polyacrylamide gel, NC membrane, filter paper, and filter pad were incubated with transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol, pH 8.3) at 25°C for 15-60 minutes. The gel sandwich was prepared as follow: the cassette was placed with the gray side down, one pre-wet fiber pad was placed on the gray side of the cassette, a sheet of filter paper was placed on the fiber pad, the equilibrated gel was placed on filter paper and the pre-wet NC membrane was placed on gel. A clean glass tube was rolled on NC membrane to remove air bubble, and the filter paper and pre-wet fiber pad were placed on the NC membrane, respectively (**Figure 29**). The gel holder cassette was placed into the electrode module and then the assembly was placed into the buffer tank containing the Bio-Ice cooling unit. The transfer buffer was filled into the buffer tank. Then, the buffer tank was put into ice-bath to decrease heat during electric running. Electricity (100 Volts) was applied for 90 minutes. The gel sandwich was disassembled; the protein-containing NC membrane was removed and air-dried at 25°C. The NC membrane can be stored at -20°C or subjected directly to the immuno-detection.

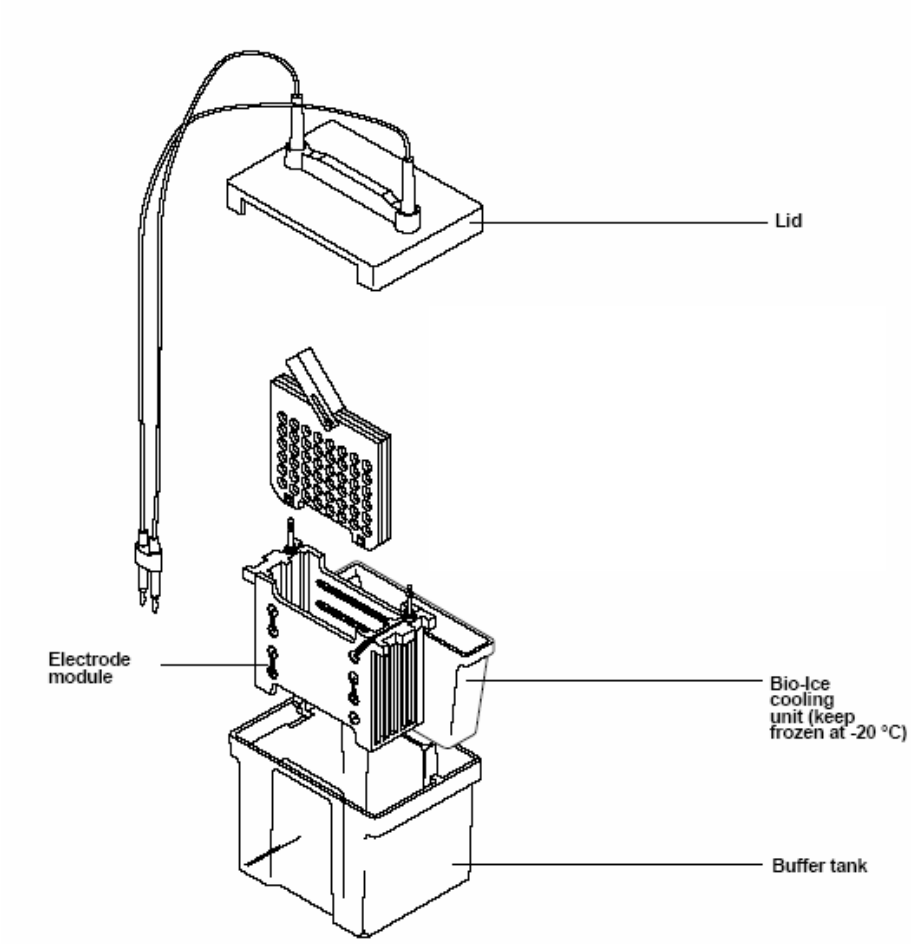

Figure 28 Equipment of the Mini Trans-Blot® Electrophoretic Transfer Cell

Source: Bio-Rad, USA

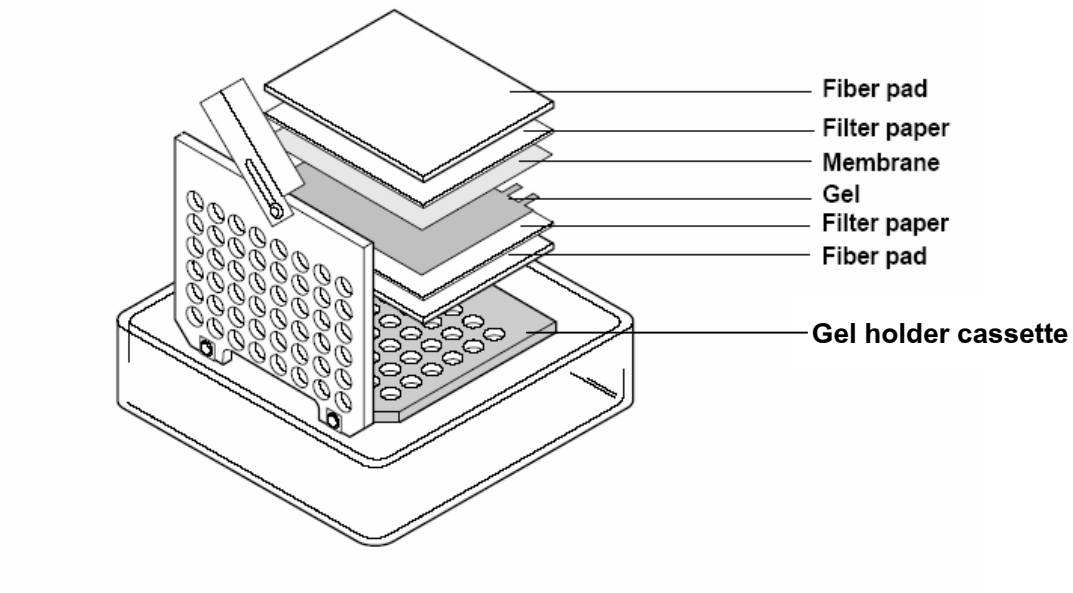

Figure 29 The gel sandwich preparation for trans-blotting

Source: Bio-Rad, USA

8. Characterization of the crude and the purified protein components of *N. kaouthia* venom

8.1 Median lethal dose (LD50) of the crude *N. kaouthia* venom

As mentioned previously, the animal experiments were performed following the guideline of the National Research Council of Thailand (NRCT) and were approved by the Ethic Committee of the Faculty of Allied Health Sciences, Thammasat University, Thailand. The LD50 of the crude venom was performed following the World Health Organization (WHO) protocol (WHO Technical Report). Male ICR mice, 5 weeks old, were purchased from the National Laboratory Animal Center, Mahidol University, Salaya Campus, Nahkon-Pathom province, Thailand. They were caged in the animal facility at the Faculty of Allied Health Sciences, Thammasat University. Mice were divided into six groups of five mice, *i.e.*, groups 1-6. Individual mice of groups 1-5 were injected intra-peritoneal with varying concentrations of the cobra venom in 200 μl of sterile PBS, *i.e.*, 5, 4, 3, 2, and 1 μg, respectively. Control mice of group 6 received 200 μl of the PBS as mock. Morbidity and mortality of the mice in all groups were observed every hour post-injection. The experiments were terminated at 24 hours post-injection. Triplicate experiments were performed. The LD50 of the venom was calculated according to the method of Reed and Muench (1938).

In order to simulate the natural snake bite situation, the venom of the above indicated amounts were dissolved in 50 μl of PBS and the intramuscular route was used instead of the intra-peritoneal route. Morbidity, mortality, and LD50 of the venom were similarly monitored.

8.2 Lethal activity of the cobra venom fractions obtained from ion exchange column chromatography

Protein fractions collected after separation of the crude cobra venom by means of ion exchange column chromatography were individually prepared at the amount of total protein equal to 0.5 LD50 of the crude venom. Each protein fraction was injected into individual 5 weeks old, male ICR mice (2 mice for each protein fraction). Morbidity and mortality of the mice within 24 hours post-venom injection were recorded in order to determine both the lethal and the non-lethal venom fractions. The

venom fractions that caused death in the mice were used as the antigen in the phage-bio-panning to select phage clones displaying human single chain variable fragments (HuScFv) for further production of the HuScFv that could bind to the respective lethal venom components.

9. Selection of phage clones displaying HuScFv that bound to the venom components in the individual fractions obtained from ion exchange column chromatography

9.1 Immobilization of the antigens

Individual wells of an ELISA plate (EIA/RIA high protein binding affinity, Corning Incorporated , NY, USA) were coated with one μg of the venom protein dissolved in 100 μl of carbonate-bicarbonate buffer, pH 9.6 (designated coating buffer). The plate was kept at 4°C overnight. Wells coated with BSA served as controls.

9.2 Phage bio-panning

9.2.1 Blocking of unbound sites in the ELISA wells

The unbound venom components were washed away from individual ELISA using a washing buffer (PBS pH 7.4 containing 0.05% Tween-20; PBST); then each well was filled with 300 μl of a blocking solution (PBS containing 3% skim milk). The plate was kept at 25°C for one hour and then washed with the washing buffer.

9.2.2 Phage binding to the immobilized antigens

The phage library from **Section 2** was diluted with equal volume of the blocking solution and 100 μl aliquots of the phage preparation (~ 10^{11} colony forming units; cfu) were added to individual antigen coated wells. The plate was kept at 25°C for 1 hour with occasional mixing the content in each well. Unbound phages were removed by extensive washing (9-10 times) with PBST.

9.2.3 Phage elution and transduction of HB2151 *E. coli*

Phages that bound to the immobilized antigen (display HuScFv to the antigen) in individual wells of the **Section 9.2.2** were eluted out by adding 50 μl of 0.5 M HCl-glycine, pH 2.2. The plate was kept at 25°C for 5 minutes. The eluted phage solution was added into 1.5 ml-plastic tube containing 3 μl of 3 M Tris-base. One hundred μl of log phase competent HB2151 *E. coli* were added and mixed with the phage solution and the preparation was kept at 25°C for 20 minutes to allow phage penetration into the HB2151 *E. coli*. The preparation was spread onto 2x YT-AG plates and the plates were incubated at 37°C overnight.

9.3 Screening of HB2151 *E. coli* transformants harboring recombinant *huscFv*-phagemids

Well isolated transformed HB2151 *E. coli* colonies on the selective plates of **Section 9.2.3** were randomly selected and used as template for PCR amplification of the *huscFv* insert in the recombinant phagemids contained in the cell of individual *E. coli* colonies. The oligonucleotide primers used in the PCR for *huscFv* amplification were: forward (*R1*) and reverse (*R2*) primers as described in **Section 2.1**. The selected HB2151 *E. coli* colonies (positive for *huscFv* amplicons at ~1,000 bp) were streaked onto a 2x YT-AG replica plate, grown at 37°C overnight. The bacteria were subsequently tested for their ability to produce HuScFv protein.

9.4 Screening for HB2151 *E. coli* harboring the recombinant *huscFv*-phagemid for their ability to produce soluble HuScFv proteins

9.4.1 Small scale HuScFv expression/preparation

Single colonies of *huscFv*-phagemid containing HB2151 *E. coli* colonies were picked from 2x YT-AG replica plate, inoculated individually into 1 ml of 2x YT-AG broth and incubated at 37°C with shaking aeration overnight. The overnight broth was added into 10 ml of fresh 2x YT-AG broth, incubated, and then centrifuged to collect the bacterial cells. The cells were resuspended in 0.5 ml of PBS or added with 0.5 ml of DW and subjected to ultra-sonication. After the cell debris was removed by centrifugation, the bacterial lysate (supernatant) thought to contain the HuScFv was proceeded to Western blot analysis for detecting the presence of the HuScFv.

9.4.2 Western blot analysis for detecting HuScFv

The bacterial lysate from **9.4.1** was subjected to 12% SDS-PAGE. The separated components in the gel were transblotted onto a nitrocellulose membrane (GE Healthcare, UK) as described in **Section 7**. The HuScFv on the NC blot was detected by using mouse anti-E-Tag monoclonal antibodies (Amersham Bioscience, USA), goat anti-mouse immunoglobulin (H & L chain)-HRP conjugate (DAKO, Denmark), and HRP substrate, respectively. Details of the Western blot analysis are given below.

The unoccupied sites on the NC blot were blocked by placing the NC in a blocking solution (5% skim milk in PBS) at 25°C for 30 minutes, washed with the washing buffer and allowed to react with 1:3,000 mouse anti-E-Tag monoclonal antibody solution at 25°C for 1 hour. The membrane was thoroughly washed and then incubated in a solution of goat anti-mouse immunoglobulin-HRP conjugate (diluted 1:3,000) at 25°C for 1 hour. After rinsing the NC to remove the antibody-enzyme conjugate, the NC was placed in a substrate solution, *i.e.*, 0.03% H_2O_2 in 2,6-dichloroindophenol [DCIP, Sigma Chemical Co., USA] at 25°C for 5 minutes, rinsed with distilled water and air-dried.

The HuScFv produced by the transformed HB2151 *E. coli* colonies were subjected to indirect ELISA for testing their binding activity to the cobra venom.

9.5 Indirect ELISA of checking binding activity of the HuScFv to the venom

One hundred μl of carbonate-bicarbonate buffer, pH 9.6 (coating buffer) containing 5 μg of crude *N. kaouthia* venom were added to each well of an ELISA plate. Control antigen well contains 100 μl of 3% skim milk and blank well contained 100 μl of PBS were included in the same plate. After keeping the plate at 37°C for 1 hour or at 4°C overnight, all wells were washed with the washing buffer, blocked with 300 μl of 3% skim milk for 1 hour, rewashed and individual wells were added with HB2151 *E. coli* lysates containing HuScFv diluted 1:2 with the washing buffer. The presence of the HuScFv that bound to the immobilized venom in the ELISA well was detected after washing away the unbound materials by adding mouse anti-E-Tag monoclonal antibody and goat anti-mouse immunoglobulin (H & L chain)-HRP conjugate. Color reaction was performed by using ABTS substrate solution (Vector Laboratories, USA) containing 2,2'-azino-bis-(3-benzthiazoline-6-sulfonic acid) and

0.03% H_2O_2. OD of the content of each ELISA well was measured against the blank at A_{405nm}. Absorbance higher than 0.05 was considered positive indirect ELISA, and the *E. coli* lysates yielded the OD higher than two-times the OD of the skim milk control were selected for further experiments.

9.6 Dot-ELISA for testing the binding of HuScFv to venom proteins

Lysates of HB2151 *E. coli* that could express crude venom bound-HuScFv (as tested by the indirect ELISA) were prepared as previously described (**Section 9.4.1**). For dot-ELISA, 3 μl of antigen (*e.g.*, crude venom, venom proteins in eluted fractions of the ion-exchange column chromatography, etc.) was dotted onto an NC square. After air-dried, the empty sites on the NC were blocked by using 5% skim milk. The venom protein(s) in the dot was allowed to react with HuScFv in the bacterial lysate (diluted 1:2 in washing buffer) at 25°C for 1 hour and at 4°C overnight. After washing, the antigen-HuScFv reactive spot was revealed by incubating the NC with mouse anti-E-Tag, goat anti-mouse immunoglobulin-alkaline phosphatase (AP) conjugate (DAKO, Denmark) and AP substrate, *i.e.*, BCIP/NBT phosphatase substrate (KPL, USA), respectively. Venom protein-HuScFv reactive spot was seen as a colored spot which was clearly different from the control spot dotted with the same antigen but was not incubated with the HuScFv or incubated with un-related HuScFv.

9.7 Western blot analysis for detecting venom components bound by the HuScFv prepared from HB2151 *E. coli* transformants

For detecting antigenic specificity of individual HuScFv of a given HB2151 transformed *E. coli* clone, Western blot analysis was performed. The crude venom of *N. kaouthia* was separated by 15% SDS-PAGE under non-reducing condition (without β-ME) and the separated components were transblotted onto the NC. After blocking the NC with 5% skim milk, the membrane was stripped vertically. Individual strips were incubated with lysates of HB2151 *E. coli* at 25°C for 1 hour and at 4°C overnight. The NC was thoroughly washed and placed in 1:3,000 diluted mouse anti-E-Tag. The antigen-antibody reactive bands were revealed by using goat anti-mouse immunoglobulin-alkaline phosphatase conjugate and phosphatase substrate.

The HuScFv of *E. coli* clones that bound to crude *N. kaouthia* venom as detected by indirect ELISA, dot-ELISA and/or Western blot analysis were produced in large scale for their mimotope searching and testing of their venom neutralizing activity *in vivo*.

10. Large scale production of soluble HuScFv from transformed HB2151 *E. coli*

10.1 Preparation of HB2151 *E. coli* lysate

Single colonies of the HB2151 *E. coli* transformants were picked from the 2x YT-AG replica plate and individually cultured in 10 ml 2x YT-AG broth at 37°C with shaking aeration overnight. Twenty-five ml of the overnight culture were added into 250 ml of fresh 2x YT-AG broth and incubated for 1 hour. The bacterial cells were harvested, added to 250 ml of 2x YT-AI broth containing 1 mM IPTG and the culture was incubated at 37°C with shaking for 3 more hours. The cell pellet was resuspended with TES, pH 8.0 buffer (5 ml) and then with 1:5 diluted TES buffer (7.5 ml). The cell suspension was kept in an ice-bath for one hour and then was subjected to ultra-sonication. HuScFv in the lysate was purified using anti-E-Tag affinity column.

For HuScFv purification by using anti-E-Tag affinity column, the pH of the *E. coli* lysate was adjusted to 7.0-8.0. The insoluble material in the preparation was removed by filtering the solution through a 0.45 μm low protein-binding filter membrane or by centrifugation at 12,000 x *g*, 4°C for 20 minutes. The preparation was ready for loading into the anti-E-Tag column.

10.2 Preparation of anti-E-Tag affinity column

The pre-packed anti-E-Tag affinity column (GE Healthcare, Uppsala, Sweden) stored in binding buffer containing preservative (0.02 M phosphate buffer, 0.05% NaN_3, pH 7.0) was re-equilibrated with 25 ml of the binding buffer without sodium azide. The buffer was applied *via* a 10-ml syringe at a flow rate of one drop per second.

10.3 Purification of the HuScFv

The prepared bacterial lysate of **Section 10.1** was applied into column at one drop per second. The flow-through solution was recycled into the column ~10 times. There after, the unbound protein was collected, and the column was washed with the

binding buffer without sodium azide until the OD of the eluate at A_{280nm} was zero (no protein).

The column bound proteins were eluted by using 15 ml of an elution buffer (0.1 M glycine, pH 3.0). One ml fractions were collected into individual 1.5 ml-plastic tubes (labeled E1-E15) containing 100 µl of 1 M Tris-base, pH 8.2 and mixed.

The column was re-equilibrated with 25 ml of the binding buffer without sodium azide and it was either re-used or stored in binding buffer containing 0.05% sodium azide at 4°C for further use.

10.4 Detection of fraction containing HuScFv

The E1-E15 fractions were monitored for the presence of protein by spectrometry at A_{280nm}. Fractions with significant OD were subjected to 12% SDS-PAGE and Western blot analysis using mouse anti-E-Tag monoclonal antibody as a detection reagent as described above (**Section 9.4.2**).

HuScFv, *i.e.*, the presence of an anti-E-Tag bound-band at ~*Mr* 27, were pooled. Total protein content of the pool was determined using Bradford reagent and the preparation was kept in small aliquots at -80°C for further use in the mimotope searching and *in vivo* venom neutralization test.

11. Mimotope searching

11.1 Bio-panning of the HuScFv with a phage display peptide library

The 12-mer peptide displayed on M13 phage [Ph.D.-12™ Phage Display Peptide Library Kit (NEW ENGLAND BioLabs, MA, USA)] was used to identify peptide sequence (mimotope) which could interact with the HuScFv.

The purified HuScFv (1 µg) was immobilized on wells of an ELISA plate, incubated at 4°C overnight. After washing away the excess (unbound) protein by using 0.1% Tween-20 in TBS, pH 7.5 (TBST), the blocking solution, *i.e.*, 0.5% BSA in TBS, pH 7.5 (300 µl) was added and incubated at 4°C for 1 hour. After a washing step, 100 µl of peptide-displaying phages (diluted 1:10 in TBST) which contained ~1.5 x 10^{11} pfu, was added into wells of ELISA plate and incubated at 25°C for 1 hour. After ten times of washing step, HuScFv-bound phages were eluted by using 0.1% BSA in 0.2 M glycine-HCl, pH 2.2. The bound phages were then amplified by infecting 20 ml of early log-phase ER2738 *E. coli* (OD at A_{600nm} ~0.3-0.4). After

removing the bacterial cells, the phages in supernatant were precipitated by using PEG/NaCl. The phages in pellet were resuspended in TBS and used in the second round of bio-panning. The bio-panning was performed repeatedly altogether three times.

After the third round of the bio-paning, the eluted phages were diluted at 10^{-2}-10^{-4} and used to infect ER2738 *E. coli* in top agarose on LB agar plate containing IPTG and X-gal. Well-isolated blue plaques were randomly picked, inoculated into 1 ml of LB broth and incubated at 37°C with shaking 250 rpm for 4.5 hours. After removing the bacterial cells, the phage-containing supernatant was used for phage genomic DNA extraction.

11.2 M13 phage genome extraction and DNA sequencing

The phage genome was extracted by using phenol/chloroform as previously described for DNA extraction (**Section 2.7.3**). The DNA pellet was dissolved in DW, and DNA preparation was subjected to 0.8% agarose gel electrophoresis, ethidium bromide staining, and visualized by UV-transilluminator before DNA sequencing.

12. *In vivo* venom neutralization test

To determine the venom neutralizing activity, purified HuScFv derived from individual phage clones were tested for their venom neutralizing activity *in vivo* individually using 5 weeks old male ICR mice as a model of envenomation. Two different protocols were used.

12.1 WHO protocol

Venom (1.5 LD50) was mixed with either purified HuScFv (10x 1.5 LD50 w/w) or horse anti-venom (10x 1.5 LD50 w/w) in 200 μl of NSS. The mixture was incubated at 37°C for 30 minutes before injected intraperitoneally into each mouse.

12.2 Modified WHO protocol

Individual mice were intraperitoneally injected with the mixture of venom (1.5 LD50) and purified HuScFv or horse anti-venom (50x 1.5 LD50) dissolved in 200 μl of NSS. Every 10 minutes thereafter, each mouse was injected with either purified HuScFv or horse anti-venom (10x 1.5 LD50) in 100 μl of NSS given *via* intraperitoneal route. Either the purified HuScFv or horse anti-venom (10x 1.5 LD50) was repeatedly injected *via* intraperitoneal route for a total of five times.

Six mice were used for each experimental group. Control mice (group 1) received only venom. Test groups received the purified HuScFv of individual clones. Positive control group received horse anti-venom. All groups of mice receive the same amount, the same volume, and the same route. Mortality of mice was observed every 20 minutes post injection until all mice in group 1 had died. Mice of the other groups were observed for one more hour before the experiments were terminated. The survival rate of mice was calculated.