CHAPTER V

RESULTS

1. Construction of human antibody phage display library

1.1 Human immunoglobulin (Ig) genes

The human Ig genes were derived from two sources as mentioned in **Section 1.1** of **Chapter IV**.

1.1.1 PBMC of young adult Thai volunteers

Demographic data of the 50 volunteers are shown in **Table 3**. Seven subjects (14%) had blood group A, 16 subjects (32%) had blood group B, 5 subjects (10%) had blood group AB, and 18 subjects (36%) had blood group O. Four subjects (4%) did not know of their own blood groups. Total amounts of RNA extracted from PBMC derived from 25 ml of each blood sample and the RNA purity (please see **Section 1.2** below) are also shown in **Table 3.**

1.1.2 Buffy coat samples of the Thai blood donors

The buffer coat volumes, blood groups of the respective donors, amount of total RNA extracted from each sample and the RNA purity are shown in **Table 4**. Each buffer coat sample was from 250-300 whole venous blood. The blood samples were negative for VDRL test, HBV surface antigen, anti-HBC virus, anti-HIV, and HIV antigen. Five buffy coat samples (50%) were from donors of blood group A, 4 samples (40%) were from blood group B donors, and 1 sample (10%) was from a blood group AB donor. As mentioned in **Section 2.2** of **Chapter IV**, the PBMC were separated from the residual red blood cells and polymorphonuclear cells (neutrophils and eosinophils) by using Ficoll-Paque (Amersham Biosciences, Sweden).

Figure 30 illustrates locations of the PBMC prepared by using VACUTAINER® CPT Cell Preparation tubes **(Figure 30A)** and Ficoll-Paque **(Figure 30B).**

Table 3 Demographic data of the 50 blood donors and the concentrations and purity of their total RNA preparations.

No.	Sex	Age	Place of birth	Blood group	Total RNA concentration (μg/ml)	RNA purity (OD at $A_{260nm}/A_{280\ nm}$)
1	M	26	Bangkok	O	0.84	1.944
2	M	22	Nakhon Sithammarat	O	1.056	1.913
3	F	18	Bangkok	A	1.048	1.926
4	F	20	Phetchaboon	AB	1.6	1.770
5	F	18	Nakhon Ratchasima	A	0.704	1.796
6	F	26	Nakhon Ratchasima	B	1.064	1.602
7	M	23	Nakhon Ratchasima	B	1.552	1.603
8	F	19	Loei	UnK	0.408	1.962
9	F	19	Sukhothai	O	1.928	1.674
10	F	18	NA	B	1.104	1.769
11	F	19	NA	B	1.864	1.713
12	F	19	Phisanulok	B	1.32	1.813
13	F	19	NA	AB	1.656	1.63
14	F	18	Bangkok	B	1.496	1.67
15	M	19	Nakhon Ratchasima	O	0.696	1.977
16	F	20	Ya Sothon	A	1.768	1.727
17	F	19	Ubon Ratchathani	AB	1.416	1.863
18	F	19	Nonthaburi	O	1.664	1.625
19	F	19	Nakhon Sithammarat	A	1.656	1.656
20	F	18	Chai Nat	O	1.344	1.931
21	M	19	Saraburi	AB	0.968	1.729
22	M	19	Buriram	O	1.488	1.958

NA, not available

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Table 3 (continued)

No.	Sex	Age	Place of birth	Blood group	Total RNA concentration (µg/ml)	RNA purity (OD at $A_{260nm}/A_{280\ nm}$)
23	F	30	Centho-Vietnam	B	0.608	1.767
24	M	36	India-Tabalpur	Unk	0.968	1.921
25	M	24	NA	A	0.864	1.612
26	F	28	Bangladesh	Unk	1.472	1.586
27	F	19	Phisanulok	O	1.824	1.572
28	F	19	Maha Sarakham	O	1.344	1.527
29	F	19	Kamphaeng Phet	A	1.688	1.529
30	M	20	Nakhon Phanom	AB	1.904	1.776
31	M	19	Sisaket	O	1.376	1.686
32	M	18	Nonthaburi	O	1.328	1.824
33	M	19	Singburi	B	0.960	1.538
34	M	19	Prachuab Khiri Khan	Unk	1.248	1.576
35	M	26	Chanthaburi	B	0.96	1.579
36	M	19	Nakhon Ratchasima	B	1.408	1.692
37	M	19	Maha Sarakham	B	2.024	1.82
38	M	25	Bangkok	O	0.832	1.962
39	M	22	Bangkok	O	0.968	1.704
40	F	25	Nakhon Ratchasima	O	0.960	1.714
41	F	28	Udon Thani	B	1.064	1.705
42	M	20	Nonthaburi	B	1.792	1.750
43	M	22	Nakhon Nayok	A	1.024	1.600
44	M	21	Buriram	B	1.280	1.702
45	M	25	Suphanburi	O	1.720	1.72

NA, not available

(continued next page)

Table 3 (continued)

No.	Sex	Age	Place of birth	Blood group	Total RNA concentration (µg/ml)	RNA purity (OD at $A_{260nm}/A_{280\ nm}$)
46	F	28	Buriram	B	1.232	1.621
47	F	23	Bangkok	O	0.880	1.642
48	M	28	Bangkok	O	1.712	1.646
49	M	25	Khon Kaen	O	1.848	1.848
50	M	38	Lam Pang	B	1.832	1.770

Table 4 Details of the 10 buffy coat samples obtained from Thai blood bank, Thai Red Cross, Bangkok, Thailand.

No.	Sex	Age	Place of birth	Buffy coat volume (ml)	Blood group	Total RNA concentration (μg/ml)	RNA purity (OD at $A_{260\text{nm}}/A_{280\text{ nm}}$)
1	NA	NA	NA	50	B	3.2	1.6
2	NA	NA	NA	50	B	2.0	1.7
3	NA	NA	NA	63	A	13	1.6
4	NA	NA	NA	63	A	8.2	1.6
5	NA	NA	NA	50	B	1.7	1.7
6	NA	NA	NA	60	A	2.5	1.8
7	NA	NA	NA	63	A	7.9	1.6
8	NA	NA	NA	60	A	11.2	1.6
9	NA	NA	NA	60	B	5.7	1.6
10	NA	NA	NA	60	AB	7.8	1.6

NA, not available

1.1.3 Total RNA amounts and purity

Total RNA extracted from ~10^7 PBMC was dissolved in 50 μl of RNase-free distilled water. For the total RNA prepared from the 50 volunteers, the average amount from 25 ml peripheral blood was 1.3 μg (range 0.41-2.0 μg). Each RNA sample was dissolved in distilled water. The preparation was kept in an ice-bath during the purity checking by gel electrophoresis and ethidium bromide staining. For long term storage, the RNA was kept at -70°C.

Purity of each total RNA preparation was determined by calculating the A_{260nm} and A_{280nm} ratio. Good quality RNA should have the ratio at 1.5-2.0. The average purity of the total RNA from the 50 volunteers was 1.70 (range 1.5-1.8); thus they were in good quality **(Table 3).**

Total RNA extracted from each of the buffy coat sample (250-500 ml of whole blood) was ~6.3 μg (range 1.7-13 μg). The average purity was 1.64 (range 1.6-1.8); thus they were also suitable for further use **(Table 4).**

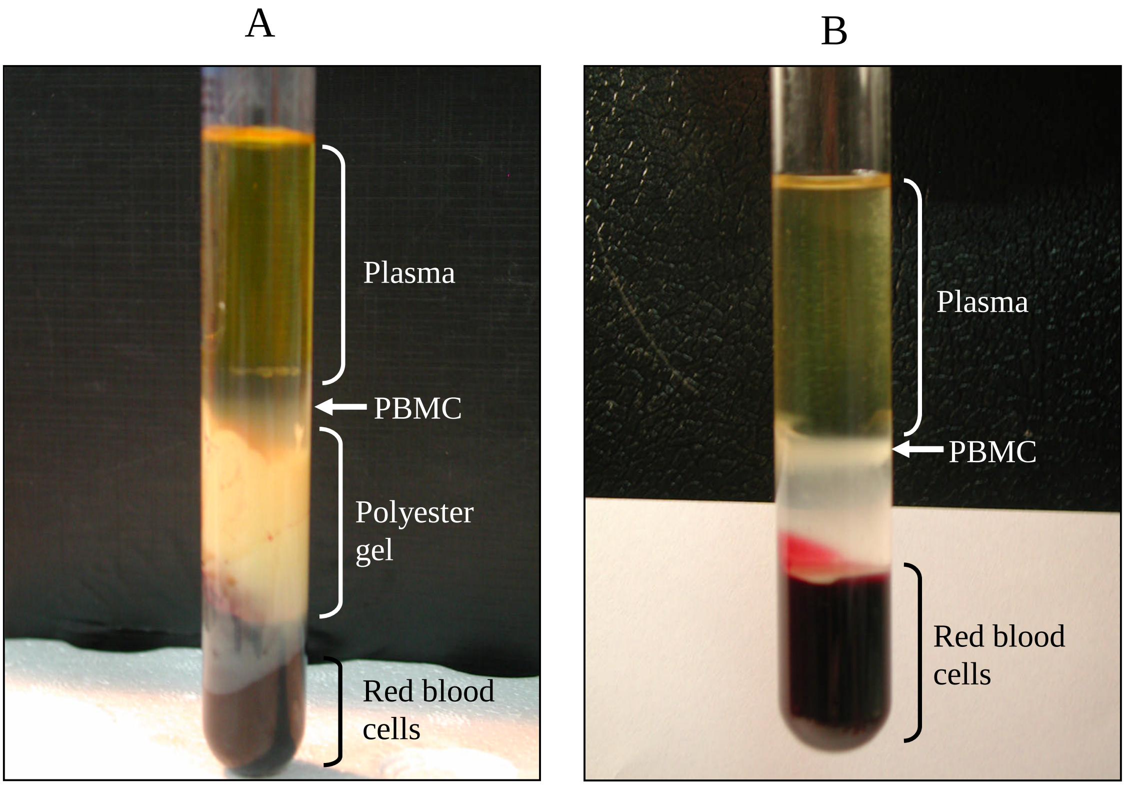


Figure 30 PBMC were prepared by using VACUTAINER® CPT™ Cell Preparation tubes containing sodium citrate (**A**) and Ficoll-Paque (**B**). Mononuclear cell layer is located between plasma and red blood cells (arrows).

1.1.4 Integrity of the RNA

Figure 31 shows integrity of representatives of total RNA prepared from the volunteers and the buffy coat samples. Each of them revealed 28 S and 18 S rRNA bands.

1.1.5 Results of DNA synthesis

Complementary DNA was synthesized from each of the total RNA preparation as described in **Section 2.5** of **Chapter IV**. Quality of all of the cDNA preparations was checked by PCR amplification of human β-actin gene segments using the cDNA as template. Amplicons of the β-actin gene segments could be obtained by the PCR implying good quality of all cDNA preparations. Examples of PCR amplification of the β-actin gene segments (~250 bp) are shown in **Figure 32**. The cDNA preparations were used as templates for amplification of human variable heavy and light chain gene segments (*VH* and *VL* sequences) for the human antibody phage library construction.

1.2 Amplification of genes encoding variable heavy (*VH*) and kappa light (*Vκ*) chains of human immunoglobulins (Ig) by PCR and preparation of *VH* and *Vκ* pools

The cDNA preparations of the 50 volunteers were pooled and divided into several small aliquots. They were used as templates for several runs of PCR for gene segment amplifications

1.2.1 *VH* amplification

Tables 5 and **6** listed nucleotide sequences used as primers in PCR amplifications of all human *VH* families. There were 14 forward primer sequences and 4 reverse primer sequences. A total number of PCR mixtures using the alternate primer pairs were 42 (14 forward primers x 3 reverse primers). **THESE PRIMERS ARE SUBJECTS OF COMPETING INTEREST IN TERMS OF PATENT RIGHT; ALL RIGHTS ARE RESERVED FOR THE NATIONAL RESEARCH COUNCIL OF THAILAND.** The sizes of *VH* amplicons were ~450 bp **(Figures 33-39).**

It was found that some primer pairs yielded nil or low amounts of amplicons, *i.e.*, combination of forward primers *VH1b, 1c, 2a, 2b, 4a, 4b, 6a,* and *7a* with reverse primer *JH1245* **(Figures 33** and **34)** when only **one** μl of the cDNA pool was used as the template in a 25 μl PCR mixtures. Thus the template amount was increased to **two** μl for each PCR mixture (*VH1b + JH1245; VH1c + JH1245; VH2a + JH1254; VH2b + JH1245; VH4a + JH1245; VH4b + JH1245; VH6a + JH1245;* and *VH7a + Jh1245*) which yielded higher amounts of the PCR amplicons, as shown in **Figures 35,** respectively, compared with those in **Figures 33** and **34.**

Amplicons at ~450 bp of the 14 forward primers paired with the *JH3* are shown in **Figures 36** and **37.** **Figures 38** and **39** illustrate amplicons of the 14 forward primers and *JH6* reverse primer. **One** μl of each primer was used in the 25 μl PCR mixture.

1.2.2 *Vκ* amplification

Thirteen degenerate forward and two degenerate reverse primers were used for amplification of human Vκ genes of all families. The primer sequences are shown in **Tables 34** and **35**, respectively. **THESE PRIMERS ARE SUBJECTS OF COMPETING INTEREST IN TERMS OF PATENT RIGHT; ALL RIGHTS ARE RESERVED FOR THE NATIONAL RESEARCH COUNCIL OF THAILAND.** The sizes of the *Vκ* amplicons analyzed on 1% agarose gel electrophoresis in comparison to the GeneRuler 1 kb ladder were ~400 bp **(Figure 40).**

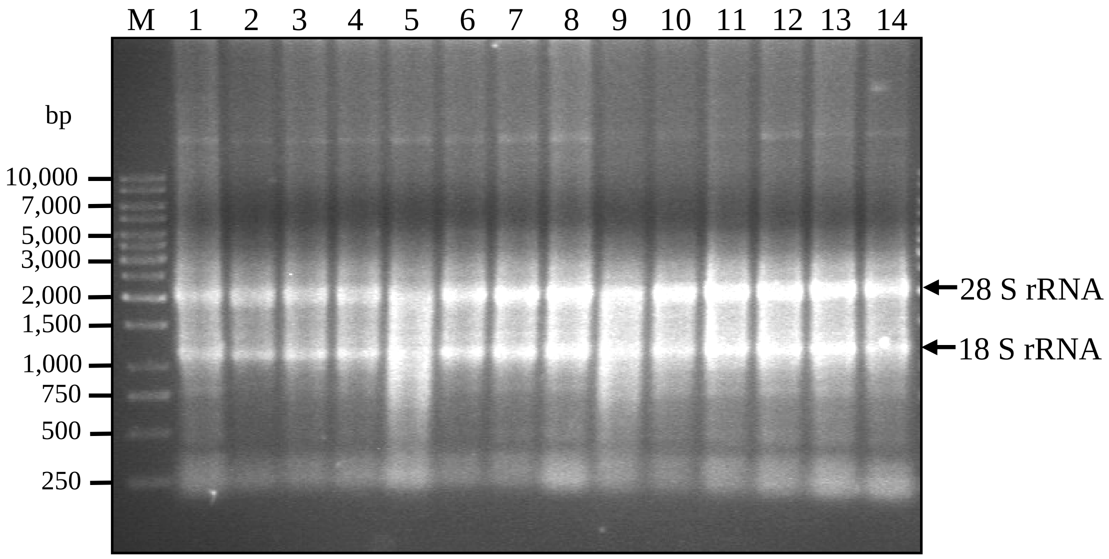


Figure 31 Agarose gel electrophoretic patterns of total RNA.

Lane M, GeneRuler 1 kb DNA Ladder

Lanes 1-10, total RNA of volunteers no. 1-10, respectively

Lanes 11-14, total RNA of buffy coat samples no. 1-4, respectively

Bands of the 28 S rRNA and 18 S rRNA are indicated by arrows

Numbers at the left are DNA sizes in bp

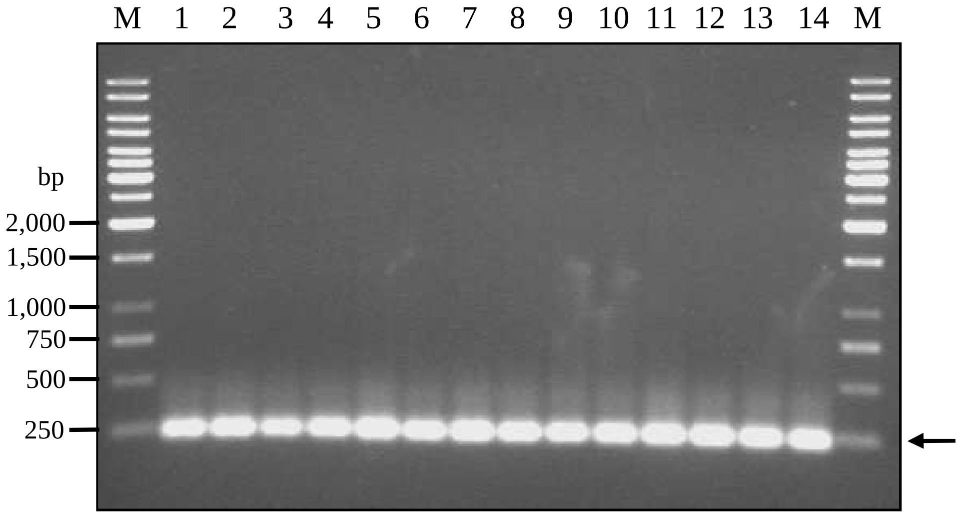


Figure 32 PCR amplicons of human β-actin gene segments (~250 bp) using cDNA of the volunteers and buffy coat samples as templates.

Lanes 1-10, amplicons of β-actin gene segments which used cDNA preparations of volunteers no. 1 to 10, respectively, as DNA templates

Lanes 11-14, amplicons of β-actin gene segments which used cDNA preparations of buffy coat samples no. 11 to 14, respectively, as DNA templates

Lanes M, GeneRuler 1 kb DNA ladder

Arrow indicates location of the β-actin gene segments

Numbers at the left are DNA sizes in bp

Table 5 Degenerate and non-degenerate forward primer sequences were designed from VBASE with *Sfi*I restriction endonuclease site (underlined alphabets) at 5'-end of the primers and were used for amplification of human *VH* by PCR.

Code	Nucleotide sequences (5' ⟶ 3')
VH1a	GGGCCCAGCCGGCCCAGGT**K**CAGCTGGTGCAG
VH1b	GGGCCCAGCCGGCCCAGGTCCAGCTTGTGCAG
VH1c	GGGCCCAGCCGGCC**S**AGGTCCAGCTGGTACAG
VH1d	GGGCCCAGCCGGCCCA**R**ATGCAGCTGGTGCAG
VH2a	GGGCCCAGCCGGCCCAGATCACCTTGAAGGAG
VH2b	GGGCCCAGCCGGCCCAGGTCACCTTGA**R**GGAG
VH3a	GGGCCCAGCCGGCCGA**R**GTGCAGCTGGTGGAG
VH3b	GGGCCCAGCCGGCCCAGGTGCAGCTGGTGGAG
VH3c	GGGCCCAGCCGGCCGAGGTGCAGCTGTTGGAG
VH4a	GGGCCCAGCCGGCCCAG**S**TGCAGCTGCAGGAG
VH4b	GGGCCCAGCCGGCCCAGGTGCAGCTACAGCAG

Bold alphabets are degenerate nucleotide

K, G or T; **S**, C or G, and **R,** A or G

All sequences are subjected to patent right

Table 5 (continued)

Code	Nucleotide sequences (5’ → 3’)
VH5a	GGGCCCAGCCGGCCGA**R**GTGCAGCTGGTGCAG
VH6a	GGGCCCAGCCGGCCCAGGTACAGCTGCAGCAG
VH7a	GGGCCCAGCCGGCCCAGGT**S**CAGCTGGTGCAA

Bold alphabets are degenerate nucleotides

R, A or G

All sequences are subjected to patent right

Table 6 Degenerate and non-degenerate reverse primer sequences that were designed from VBASE with polynucleotide linkers (underlined alphabets) at 5’-end of the primers and were used for amplification of human *VH* by PCR.

Code	Nucleotide sequences (5’ to 3’)
JH1245	AGATCCGCCGCCACCCGACCCACCACCGCCCGAGCCACCGCCACCTGAGGAGAC**R**GTGACCAGGGT
JH3	AGATCCGCCGCCACCCGACCCACCACCGCCCGAGCCACCGCCACCTGAAGAGACGGTGACCATTGT
JH6	AGATCCGCCGCCACCCGACCCACCACCGCCCGAGCCACCGCCACCTGAGGAGACGGTGACCGTGGT

Bold alphabets are degenerate nucleotide

R, A or G

All sequences are subjected to patent right

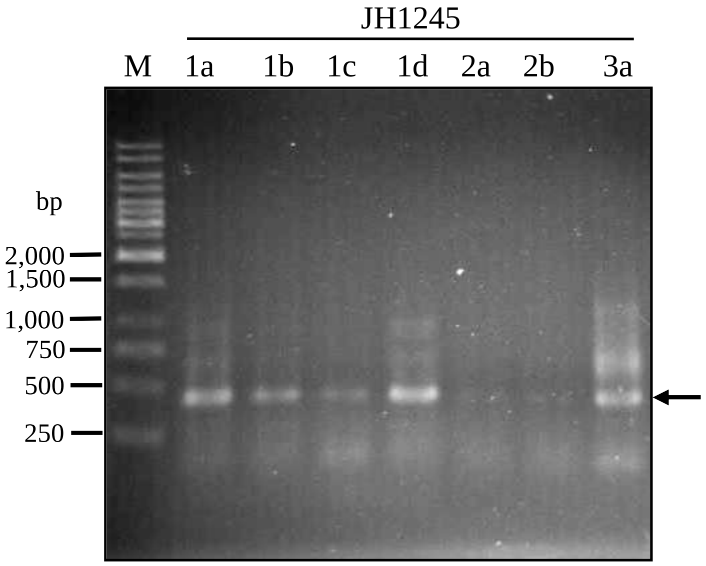


Figure 33 PCR products of VH-coding DNA sequences of some immunoglobulin subclasses.

VH1a, 1b, 1c, 1d, 2a, 2b, and ***3a*** family-specific forward primers were combined with ***JH1245*** reverse primer, specific to J gene segments 1, 2, 4, and 5 of heavy chain (*JH1245*)

Lane M, GeneRuler 1 kb DNA ladder

Each PCR product, which was amplified from **one** μl of pooled cDNA prepared from peripheral blood mononuclear cells (PBMC) of multiple blood donors, was analyzed by 1% agarose gel electrophoresis. The size of each PCR product was about ~450 bp (indicated by arrow)

Numbers at the left are DNA sizes in bp

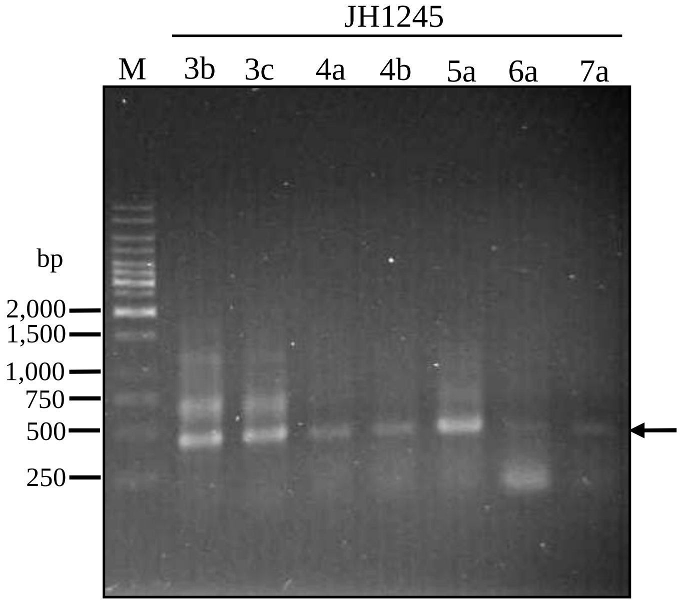


Figure 34 PCR products of VH-coding DNA sequences of some immunoglobulin subclasses

VH3b, 3c, 4a, 4b, 5a, 6a, and ***7a*** family-specific forward primers were combined with ***JH1245*** reverse primer, specific to J gene segments 1, 2, 4, and 5 of heavy chain (*JH1245*)

Lane M, GeneRuler 1 kb DNA ladder

Each PCR product, which was amplified from **one** µl of pooled cDNA prepared from peripheral blood mononuclear cells (PBMC) of multiple blood donors, was analyzed by 1% agarose gel electrophoresis. The size of each PCR product was about ~450 bp (indicated by arrow)

Numbers at the left are DNA sizes in bp

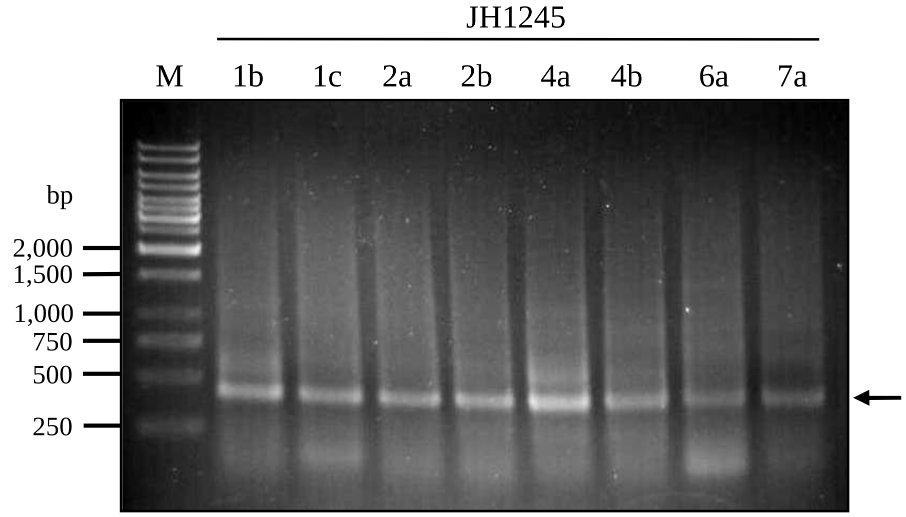


Figure 35 PCR products of VH-coding DNA sequences of some immunoglobulin subclasses.

VH1b, 1c, 2a, 2b, 4a, 4b, 6a, and ***7a*** family-specific forward primers were combined with ***JH1245*** reverse primers, specific to J gene segment of heavy chains (*JH*) of all immunoglobulin subclasses

Lane M, GeneRuler 1 kb DNA ladder

Each PCR product, which was amplified from **two** μl of pooled cDNA prepared from peripheral blood mononuclear cells (PBMC) of multiple blood donors, was analyzed by 1% agarose gel electrophoresis. The size of each PCR product was about ~450 bp (indicated by arrow)

Numbers at the left are DNA sizes in bp

1.2.3 Preparations of *VH* and *Vκ* pools

The *VH* and *Vκ* PCR amplicons in **1.2.1** and **1.2.2** were separately pooled, and concentrated by ethanol precipitation. Proteins were eliminated by using phenol/chloroform/isoamyl alcohol (25:24:1 v/v). After suspending individual DNA pool in TE buffer, pH 8.0, the solutions were separated in 1.5% agarose (this agarose concentration gave better resolution of the DNA than using 1% agarose). **Figure 41** shows locations of *VH* and *Vκ* pools at ~450 and 400 bp, respectively.

VH and *Vκ* DNA sequences were individually purified from the gel pieces excised from the agarose slabs by using GENECLEAN® II Kit.

1.3 Generation of single chain DNA sequences (*huscFv*) encoding human VH-peptide linker-VL proteins (HuScFv)

1.3.1 Principle of SOE-PCR

The principle of SOE-PCR was described in **Section 2.8.1** of **Chapter IV**.

1.3.2 Optimization of the SOE PCR condition

A. Optimal annealing temperatures in linking the two nucleotides and the outer forward and reverse primers

For linking the two nucleotide linkers on the 3’-end of *VH* and *5’-end of Vκ* sequences together, the annealing temperatures were set at 50°C, 55.5°C, 60.8°C, and 66°C. After 10 cycles of the nucleotide linker annealing step, the outer forward and reverse primers were added into the SOE-PCR mixture. The annealing temperature for the outer primers was set at 50°C for 20 repeated cycles. The expected size of the *huscFv* amplicon was ~750 bp. It was found that there was no difference on the yield of the *huscFv* amplicons by using different temperatures for linking the two nucleotide linkers together (**Figure 42).**

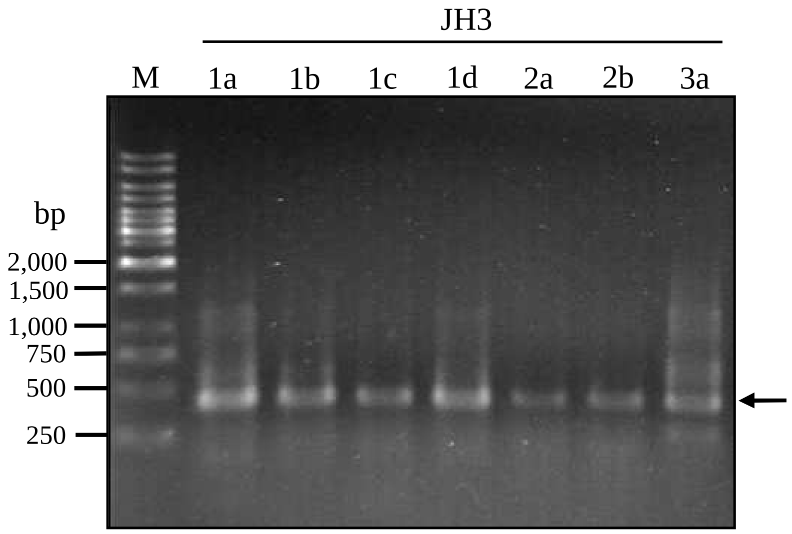


Figure 36 PCR products of VH-coding DNA sequences of some immunoglobulin (Ig) subclasses.

VH1a, 1b, 1c, 1d, 2a, 2b, and ***3a*** family-specific forward primers were combined with ***JH3*** reverse primer, specific to J gene segment 3 of heavy chain (*JH3*)

Lane M, GeneRuler 1 kb DNA ladder

Each PCR product, which was amplified from **one** μl of pooled cDNA prepared from peripheral blood mononuclear cells (PBMC) of multiple blood donors, was analyzed by 1% agarose gel electrophoresis. The size of PCR product was about ~450 bp (indicated by arrow)

Numbers at the left are DNA sizes in bp

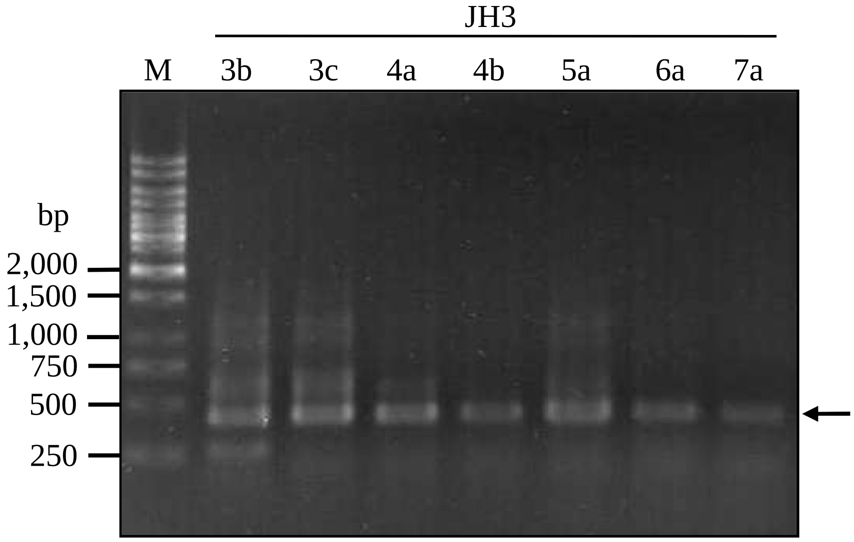


Figure 37 PCR products of VH-coding DNA sequences of some immunoglobulin (Ig) subclasses.

VH3b, 3c, 4a, 4b, 5a, 6a, and ***7a*** family-specific forward primers were combined with ***JH3*** reverse primer, specific to J gene segment 3 of heavy chain (*JH3*)

Lane M, GeneRuler 1 kb DNA ladder

Each PCR product, which was amplified from **one** μl of pooled cDNA prepared from peripheral blood mononuclear cells (PBMC) of multiple blood donors, was analyzed by 1% agarose gel electrophoresis. The size of PCR product was about ~450 bp (indicated by arrow)

Numbers at the left are DNA sizes in bp

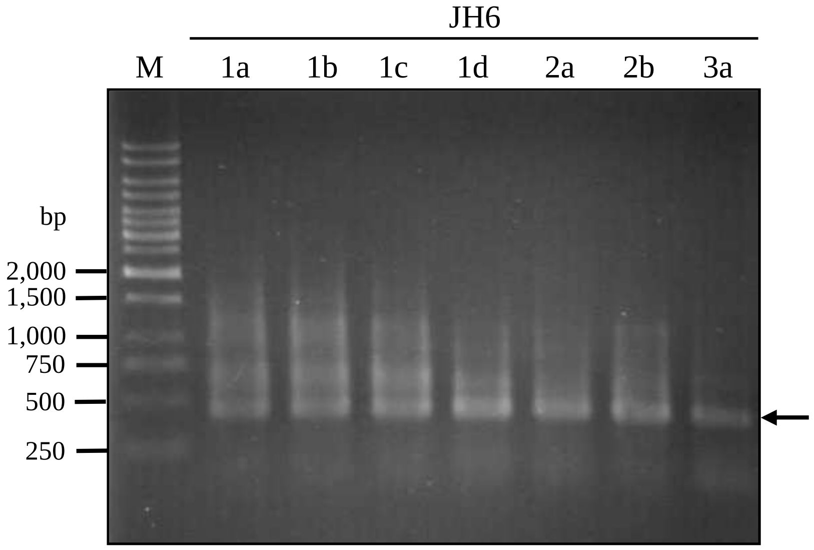


Figure 38 PCR products of VH-coding DNA sequences of some immunoglobulin (Ig) subclasses.

VH1a, 1b, 1c, 1d, 2a, 2b, and ***3a*** family-specific forward primers were combined with ***JH6*** reverse primer, specific to J gene segment 6 of heavy chain (*JH6*)

Lane M, GeneRuler 1 kb DNA ladder

Each PCR product, which was amplified from **one** μl of pooled cDNA prepared from peripheral blood mononuclear cells (PBMC) of multiple blood donors, was analyzed by 1% agarose gel electrophoresis. The size of PCR product was about ~450 bp (indicated by arrow)

Numbers at the left are DNA sizes in bp

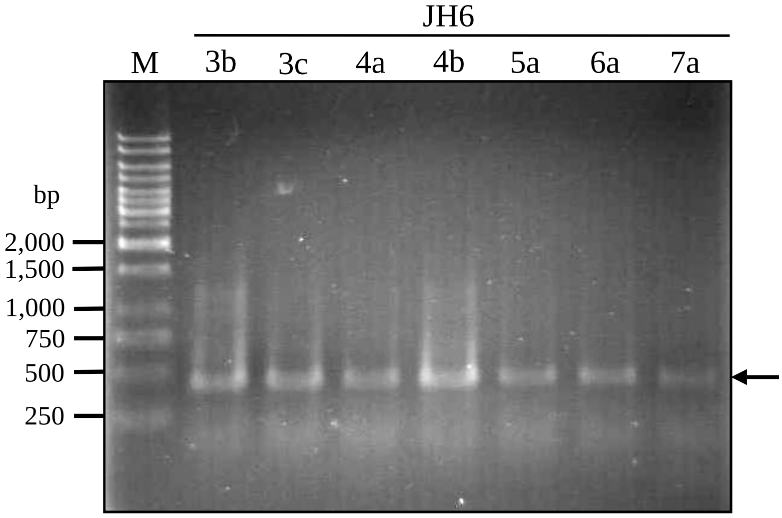


Figure 39 PCR products of VH-coding DNA sequences of some immunoglobulin (Ig) subclasses.

VH3b, 3c, 4a, 4b, 5a, 6a, and ***7a*** family-specific forward primers were combined with ***JH6*** reverse primer, specific to J gene segment 6 of heavy chain (*JH6*)

Lane M, GeneRuler 1 kb DNA ladder

Each PCR product, which was amplified from **one** μl of pooled cDNA prepared from peripheral blood mononuclear cells (PBMC) of multiple blood donors, was analyzed by 1% agarose gel electrophoresis. The size of PCR product was about ~450 bp (indicated by arrow)

Numbers at the left are DNA sizes in bp

Table 7 Degenerate and non-degenerate forward primer sequences were designed from VBASE with polynucleotide linker (underlined alphabets) at 5’-end of primers and were used to amplify human *Vκ* in PCR.

Code	Nucleotide sequences (5’→ 3’)
Vκ1a	GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCT**R**ACATCCAGATGACCCAG
Vκ1b	GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCTG**M**CATCCAGTTGACCCAG
Vκ1c	GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCTGCCATCC**R**GATGACCCAG
Vκ1d	GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCTGTCATCTGGATGACCCAG
Vκ2a	GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCTGATATTGTGATGACCCAG
Vκ2b	GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCTGAT**R**TTGTGATGACTCAG
Vκ3a	GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCTGAAATTGTGTTGAC**R**CAG

Bold alphabets are degenerate nucleotides. **R**, A or G; **M**, A or C

All sequences are subjected to patent right

Table 7 (continued)

Code	Nucleotide sequences (5’⟶ 3’)
Vκ3b	GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCTGAAATAGTGATGACGCAG
Vκ3c	GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCTGAAATTGTAATGACACAG
Vκ4a	GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCTGACATCGTGATGACCCAG
Vκ5a	GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCTGAAACGACACTCACGCAG
Vκ6a	GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCTGAAATTGTGCTGACTCAG
Vκ6b	GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCTGATGTTGTGATGACACAG

All sequences are subjected to patent right

Table 8 Degenerate and non-degenerate reverse primer sequences were designed from VBASE with *Not*I restriction endonuclease site (underlined alphabets) at 5’-end of primers and were used to amplify human *Vκ* in PCR.

Code	**Nucleotide sequences (5’→ 3’)**
JK1234	CCTGCGGCCGCTTTGAT**H**TCCA**SY**TTGGTCCC
JK5	CCTGCGGCCGCTTTAATCTCCAGTCGTGTCCC

Bold alphabets are degenerate nucleotides.

H, A, C, or T; **S**, C or G; **Y**, C or T

All sequences are subjected to patent right

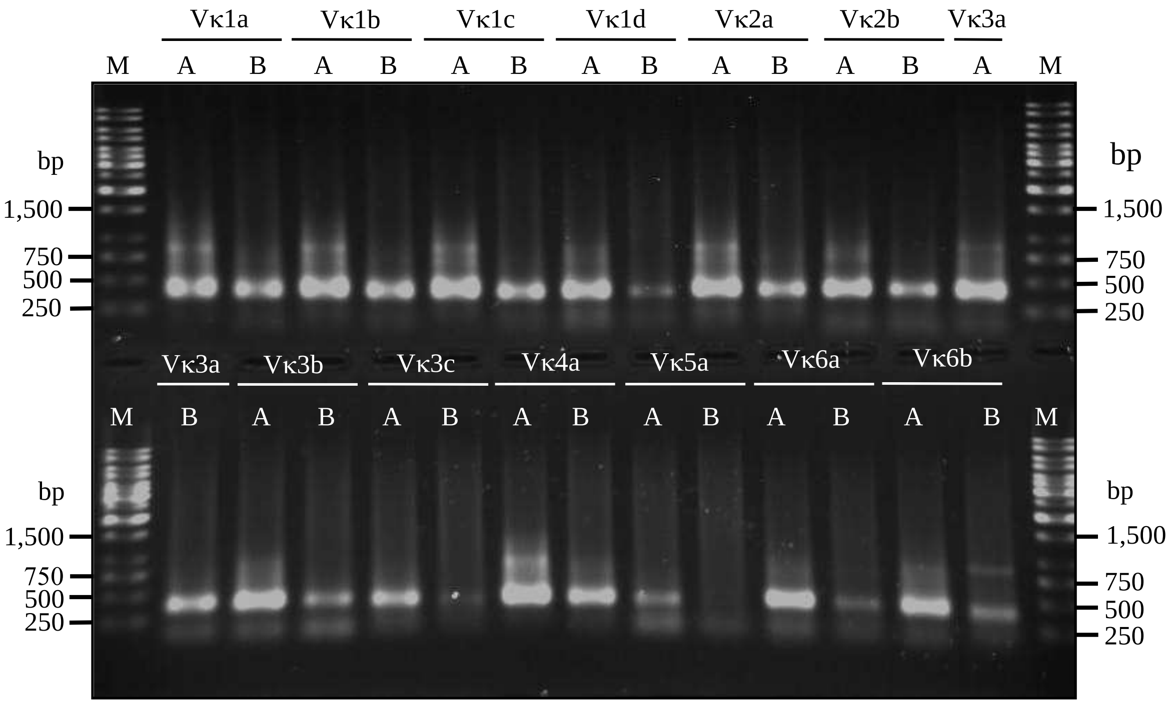


Figure 40 PCR products of human Vκ-coding DNA sequences (~400 bp).

Lanes M, GeneRuler 1 kb DNA ladder

The PCR products, which were amplicons of pooled cDNA prepared from peripheral blood mononuclear cells (PBMC) of volunteers and buffy coat samples, were analyzed on 1% agarose gel electrophoresis and stained by ethidium bromide. The size of Vκ amplicons was ~400 bp. The ***Vκ1a, 1b, 1c, 1d, 2a, 2b, 3a, 3b, 3c, 4a, 5a, 6a,*** and ***6b*** forward primers were combined with ***Jκ1234*** reverse primers (lanes A), and combined with ***Jκ5*** reverse primer (lanes B)

Numbers at the left and right are DNA sizes in bp

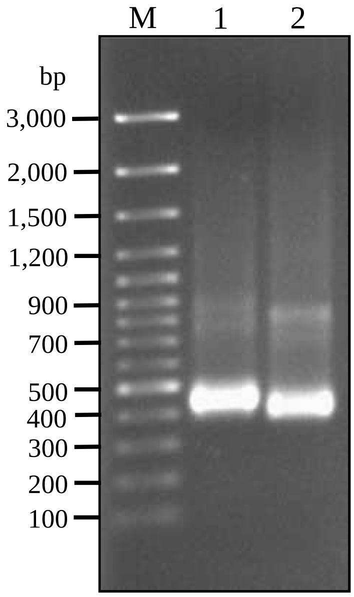


Figure 41 *VH* and *Vκ* DNA pools were analyzed on 1.5% agarose gel.

Lane M, GeneRuler 100 bp DNA ladder

Lanes 1 and 2, *VH* and *Vκ* DNA pools, respectively

Numbers at the left are DNA sizes in bp

For optimization of the annealing temperature of the outer primers to the template DNA, the nucleotide linker annealing temperature was set at 66°C but the annealing temperatures of outer primers to the DNA template were varied: 50°C, 55.5°C, 60.8°C, and 66°C. It was found that the 66°C was the optimal temperature for outer primer annealing because at this temperature the most intense band of *huscFv* amplicons was seen at ~750 bp **(Figure 43).**

B. SOE-PCR step at which outer primers were added to the PCR mixture

For testing the most appropriate step in the SOE-PCR at which the outer primers should be added to the PCR mixture, the annealing temperature of the SOE-PCR was set at 66°C. The outer primers were added at two different steps, *i.e.*, **during** or **after** the *VH* and *Vκ* linking. It was found that adding the outer primers **during** *VH* and *Vκ* linking yielded only low intensity of expected *huscFv* band (lanes 2**, Figure 44**). More intense band of *huscFv* was obtained when the outer primers were added after the *VH* and *Vκ* linking (lanes 1**, Figure 44**).

1.3.3 Preparation of *huscFv* for ligation with phagemid vector

Large amount of the *huscFv* sequences was prepared by running several PCR amplifications. **Figure 45** shows *huscFv* amplicons from 19 representatives of the PCR amplifications**.** The *huScFv* sequences contained in the DNA bands of ~750-800 bp (indicated by arrow in **Figure 45**) were extracted (**Section 2.7.3 of Chapter IV**) from a pool of agarose gel pieces excised out from the 1.5% agarose slabs.

1.4 *Sfi*I- and *Not*I-restricted digestion of the *huscFv* sequences

The *huscFv* sequences were cut with *Sfi*I and *Not*I restriction endonucleases (**Sections 2.9** and **2.10** in **Chapter IV**). The amount of cut sequences was quantified by separating the preparation in 1% agarose gel electrophoresis. The MassRuler^{TM} DNA Ladder (Fermentas, Lithuania) with known amounts of DNA was run concurrently in the same gel. After ethidium bromide staining, the amount of the cut-*huscFv* sequences was estimated by comparing intensity of the band with those of the MassRuler^{TM} DNA Ladder. Example of the agarose gel for the *huscFv* quantification is shown in **Figure 46.**

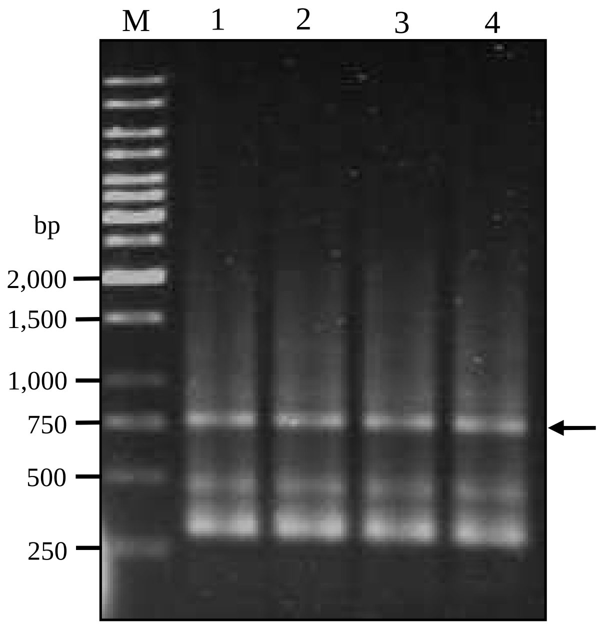


Figure 42 Optimization of annealing temperature for polynucleotide linkers.

Lane M, GeneRuler 1 kb DNA ladder

The *huscFv* sequences were generated by SOE-PCR using 50°C (lane 1), 55.5°C (lane 2), 60.8°C (lane 3), and 66°C (lane 4) annealing temperatures. PCR products were analyzed on 1% agarose gel. The size of *huscFv* amplicon was about 750 bp (indicated by arrow)

Numbers at the left are DNA sizes in bp

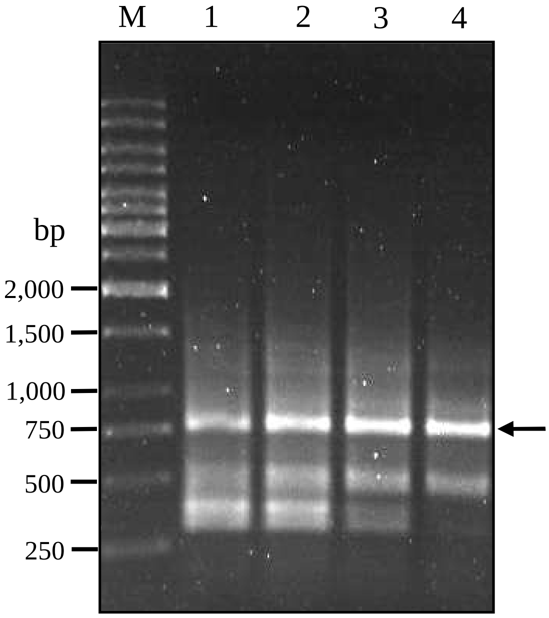


Figure 43 Optimization of annealing temperature for outer primers.

Lane M, GeneRuler 1 kb DNA ladder

The *huscFv* sequences were generated by SOE-PCR using 50°C (lane 1), 55.5°C (lane 2), 60.8°C (lane 3), and 66°C (lane 4) annealing temperatures of outer primers. PCR products were analyzed on 1% agarose gel. The size of *huscFv* sequences was about 750 bp (indicated by arrow).

Numbers at the left are DNA sizes in bp

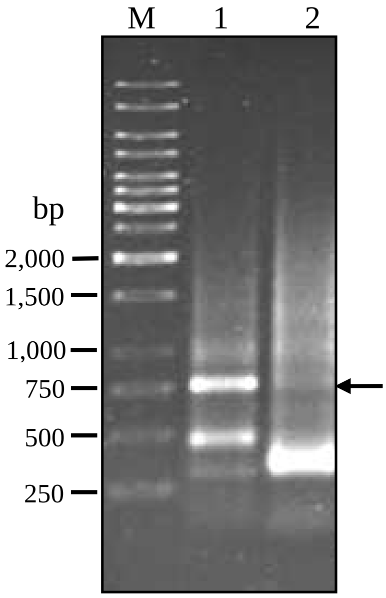


Figure 44 Influence of outer primer adding step for generation of *huscFv*.

Lane M, the GeneRuler 1 kb DNA ladder

Lane 1, the *huscFv* amplicons that was generated by adding outer primer after *VH* and *Vκ* linking

Lane 2, the *huscFv* amplicons that was generated by adding outer primer during *VH* and *Vκ* linking. PCR products were analyzed on 1% agarose gel. The size of *huscFv* amplicon was about 750 bp (indicated by arrow)

Numbers at the left are DNA sizes in bp

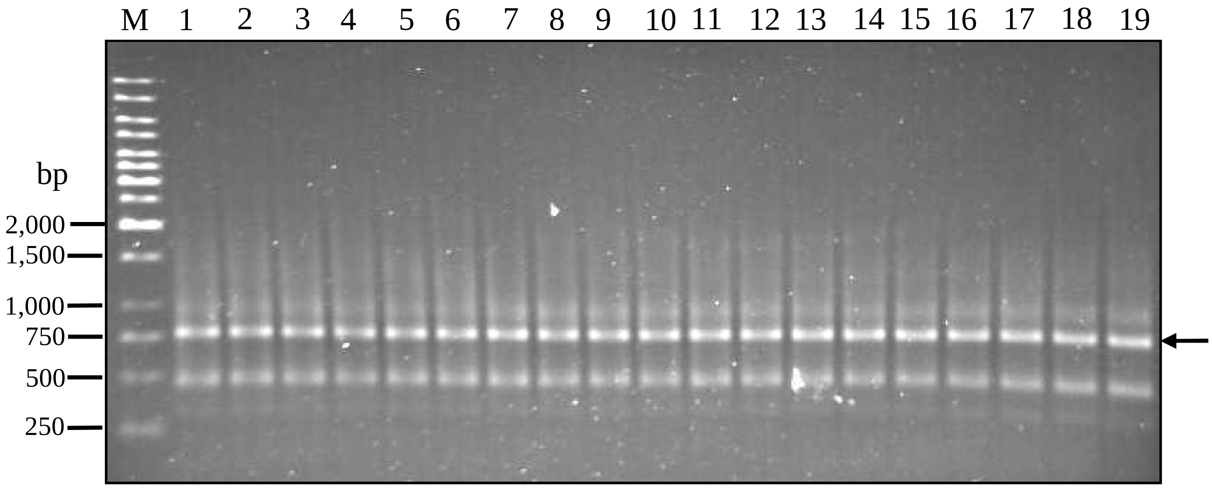


Figure 45 Large scale preparation of *huscFv*.

Lane M, GeneRuler 1 kb DNA ladder

Lanes 1-19, the 19 representative samples of SOE-PCR

Each DNA amplicon was analyzed on 1% agarose gel. The expected size of each *huscFv* amplicon was 750 bp (indicated by arrow)

Numbers at the left are DNA sizes in bp

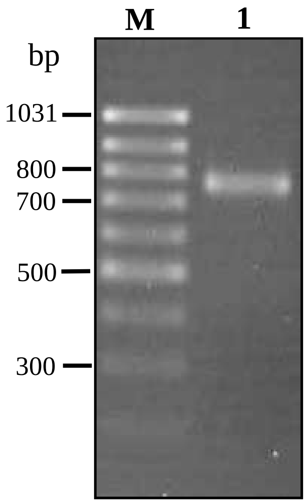


Figure 46 Quantification of *huscFv*.

Amount of *Not*I- and *Sfi*I-cut-*huscFv* (lane 1) was quantified by comparing with MassRuler DNA ladder (lane M).

Numbers at the left are DNA sizes in bp

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

The *Sfi*I- and *Not*I-cut-*huscFv* sequences (150 ng) were ligated with the pCANTAB5E phagemids pre-cut with the same enzymes using the protocol of the Expression Module/Recombinnat Phage Antibody System of GE Health Care (UK).

1.6 Preparation of recombinant *huscFv*-pCANTAB5E vector for *E. coli* transformation by using electroporation method

The recombinant *huscFv*-pCANTAB5E vector was purified from the ligation mixture (elimination of salts and proteins) by using phenol/chloroform/isoamyl alcohol (25:24:1) treatment and ethanol precipitation. The purified recombinant vectors were ready for the *E. coli* transformation.

1.7 Transformation efficiency of the electro-competent TG1 *E. coli* cells

The competent TG1 *E. coli* cells were prepared (**Section 2.13** in **Chapter IV**). The transformation efficiency of the bacterial cells [colony forming units (cfu) per μg of DNA] as tested by using 10 ng of uncut pUC19 was ~10^9 cfu/μg of uncut pUC19 (**Figure 47**).

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

The cfu of TG1 *E. coli* transformants were ~260 at 10^{-4} dilution (**Figure 48**). The total transformants from this electroporation was 2.6 x 10^8.

1.9 Phage rescuing

M13KO7 phages were used to infect the recombinant *huscFv*-pCANTAB5E-haboring TG1 *E. coli* at moi of 5:1. The progeny recombinant phage particles in culture supernatant were collected and subjected to phage precipitation using PEG/NaCl (**Section 2.16** of **Chapter 2.16**).

1.10 Determination of the phage titer

Titer of the rescued phages was determined as cfu of the phage-infected TG1 *E. coli* cells which were ampicillin-resistant and could grow on the ampicillin-containing agar plate. The titer of phage library was 6.5 x 10^{12} cfu/ml (**Figure 49**).

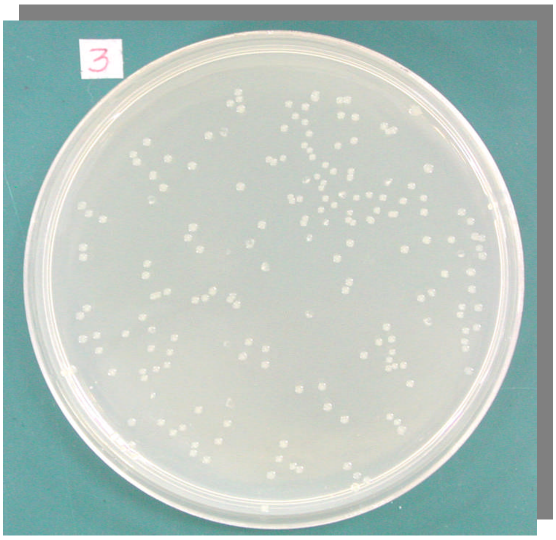


Figure 47 The transformation efficiency of electro-competent TG1 *E. coli*.

pUC19 (10 ng) was introduced into electro-competent cells. After electric pulsing and cell recovering, all transformants in cell suspension were centrifuged and spreaded on LB-A agar plate. Total numbers of TG1 *E. coli* colonies are ~170. The efficiency of this competent cell is ~10^9 cfu/µg of uncut pUC19.

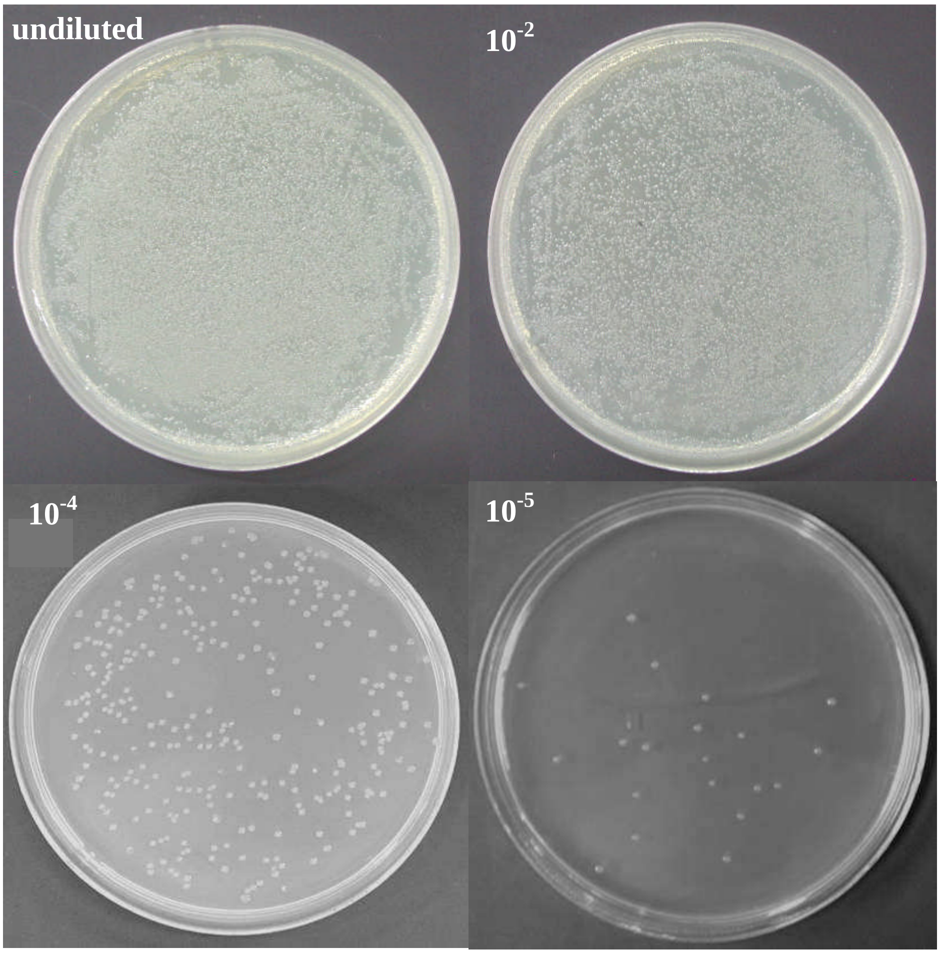


Figure 48 TG1 *E. coli* transformants on SOBAG agar plate.

Ten µl each of the four dilutions (undiluted, 10^{-2}, 10^{-4}, and 10^{-5} diluted TG1 *E. coli* transformants) of 1 ml of TG1 transformed suspension were spreaded on SOBAG agar plate. A total of ~260 colonies at 10^{-4} dilution of 10 µl of the 1-ml TG1 *E. coli* transformants were obtained. The repertoire of the phage library was 2.6×10^8 cfu.

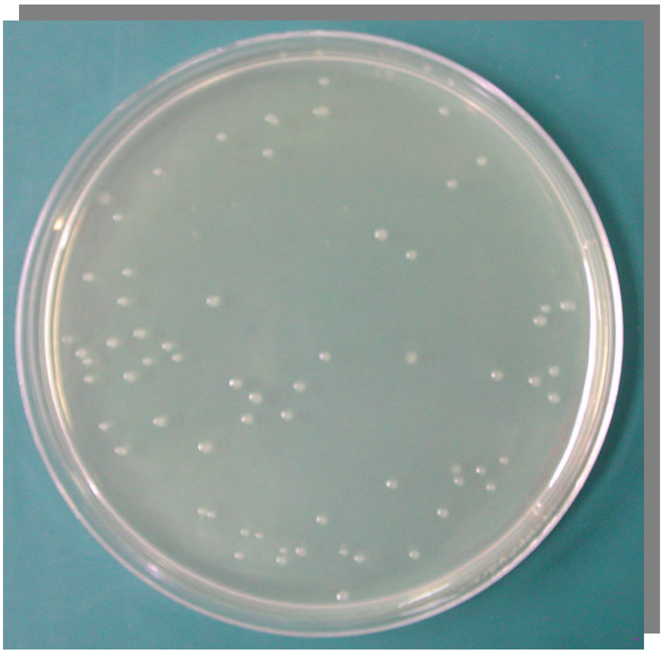


Figure 49 Phage titer of human antibody phage display library.

Various dilutions of phage library was prepared (10^7, 10^8, and 10^9 phage dilution) in sterile PBS, pH 7.4 to get appropriate number of bacterial colony (not more that 200 cfu for 100-mm ϕ standard bacterial culture plate). Recombinant phage-infected TG1 *E. coli* cells were grown on 2x YT-AG agar plate. Ten µl each of the three phage dilutions was used to infect 100 µl of log-phase TG1 *E. coli*. A total of ~65 colonies at 10^{-9}dilution of phage preparation were obtained. The titer of phages was 6.5 x 10^{12} cfu/ml.

2. Characterization of human antibody phage display library

2.1 *huscFv* amplification

The *huscFv* were amplified directly from the 20 well-isolated colonies of TG1 *E. coli* transformants that were randomly selected from SOBAG agar plate that was spread with 10 μl of 10^{-4} dilution (**Section 3.1** of **Chapter IV**). Seventeen colonies from 20 colonies (85%) of TG1 *E. coli* transformants contained *huscFv* at the size ~1,000 bp [**Figure 50**, lanes no. 1-6, 8-12, and 14 in (A) and lanes no. 16-20 in (B)]. Three colonies [**Figure 50**, lanes no. 7, 13, and 15 in (A)] from total 20 colonies (15%) of TG1 *E. coli* transformants did not contain *huscFv*.

2.2 *Mva*I RFLP of *huscFv*

For *huscFv* diversity, the different DNA banding patterns of the *Mva*I-cut *huscFv* sequences were determined. *Mva*I-cut-*huscFv* sequences, which were amplified from individual transformed TG1 *E. coli* clones no. 1-6, 8-12, and 14 (A), and 16-20 (B) in **Figure 50**, were analyzed on 12% polyacrylamide gel [lanes no. 1-6, 8-12, 14, and 16 in (A) and lanes no. 17-20 in (B) in **Figure 51**]. Fifteen different patterns of *Mva*I-cut DNA bands were obtained from 17 *huscFv* sequence tested implying high diversity of the *huscFv* sequences. There were similar patterns of *Mva*I RFLP [lanes no. 3 and 4 (°) and lanes no. 16 and 19 (*) of **Figure 51**].

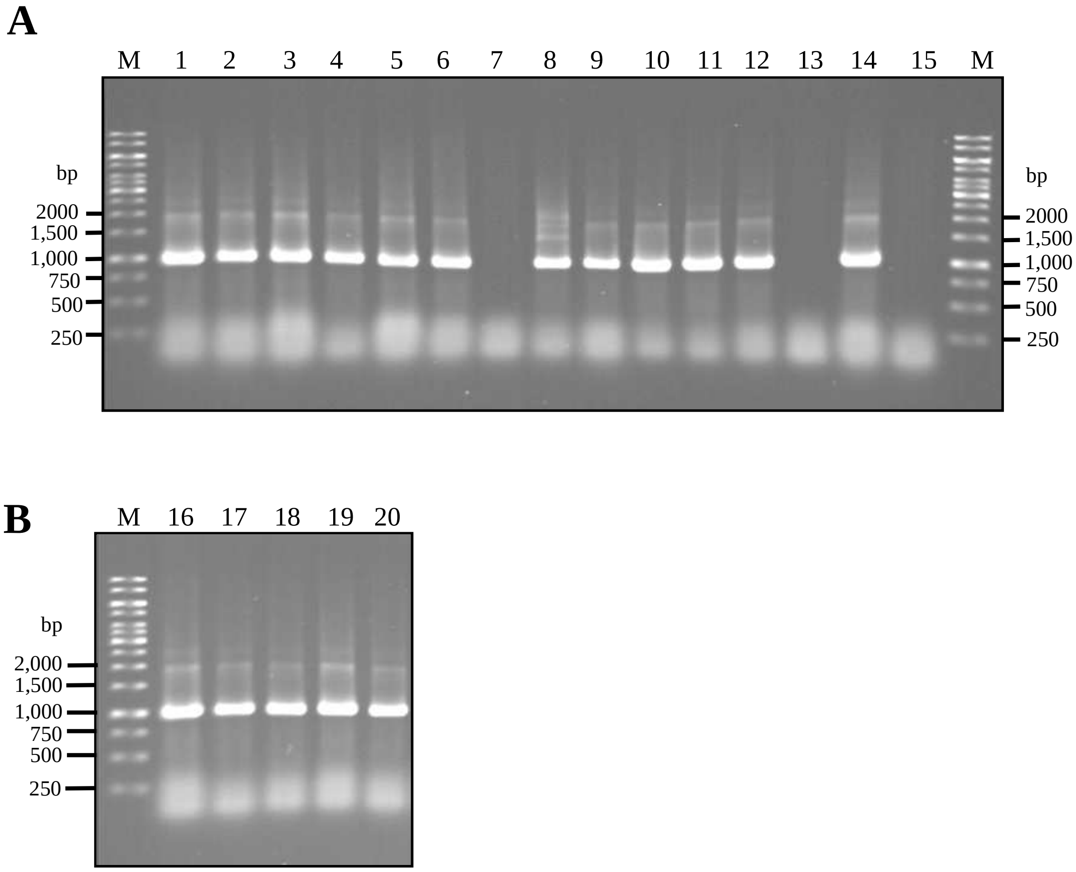


Figure 50 *huscFv* amplification of 20 TG1 *E. coli* clones derived from the transformation.

Lanes M of both (**A**) and (**B**), GeneRuler 1 kb DNA ladders

The PCR products of *huscFv* were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide. Seventeen colonies from 20 colonies (85%) of TG1 *E. coli* transformants contained *huscFv* at the size ~1,000 bp [lanes no. 1-6, 8-12, and 14 in (**A**) and lanes no. 16-20 in (**B**)]. Three colonies [lanes no. 7, 13, and 15 in (**A**)] from total 20 colonies (15%) of TG1 *E. coli* transformants did not contain *huscFv*.

Numbers of the left and right of (**A**) and at the left of (**B**) are DNA sizes in bp

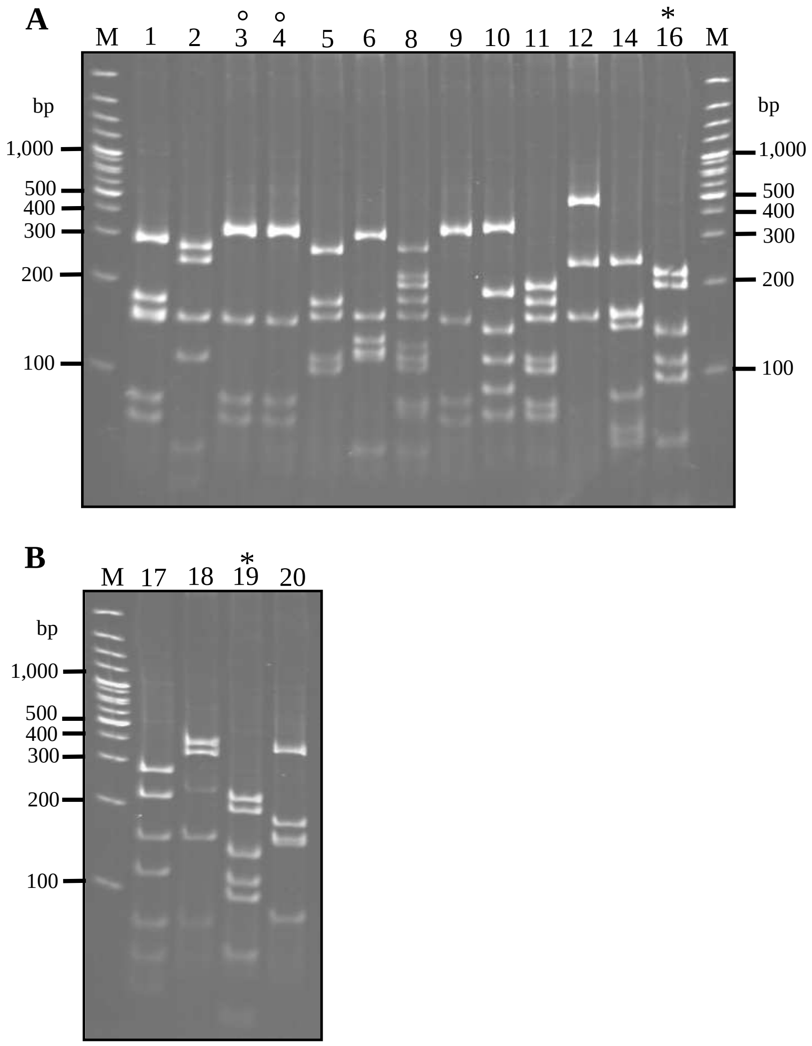


Figure 51 Patterns of *Mva*I RFLP of *huscFv* sequences.

Lanes M of (**A**) and (**B**), GeneRuler™ 100 bp DNA ladder plus

*Mva*I-cut-*huscFv* sequences, which were amplified from individual transformed TG1 *E. coli* clones no. 1-6, 8-12, and 14 (**A**), and 16-20 (**B**) in **Figure 50**, were analyzed on 12% acrylamide gel [lanes no. 1-6, 8-12, 14, and 16 in (**A**) and lanes no. 17-20 in (**B**)].

Numbers of the left and right of (**A**) and at the left of (**B**) are DNA sizes in bp

3. Proteomic study of *N. kaouthia* venom

3.1 SDS-PAGE of crude *N. kaouthia* venom

The protein components of crude *N. kaouthia* venom obtained from Queen Saovabha Memorial Institute (QSMI), Thai Red Cross, Bangkok, Thailand, was analyzed on 15% polyacrylamide gel under non-reducing and reducing condition and stained with Coomasie™ Briliant Blue R-250 as shown in **Figure 52**. The SDS-PAGE in non-reducing condition and protein staining revealed that the venom of *N. kaouthia* contained proteins ranging in their relative molecular masses (*Mr*) from ~7 to >204 kDa which could be arbitrarily grouped into four groups, *i.e.*, <20 kDa, >20 to ~30 kDa, >40 to ~90 kDa, and >204 kDa (**Figure 52A**). Under reducing condition, cobra protein components are seen between ~7 to 116 kDa; many of the bands seen under the non-reducing conditions have disappeared (**Figure 52B**).

3.2 The proteome of *N. kaouthia* venom identified by 2DE-based-liquid chromatography/tandem mass spectrometry (2DE-LC/MS-MS)

Figure 53 (upper panel) illustrates the 2DE-proteome of the *N. kaouthia* venom. Twenty-four protein spots were visualized after protein staining with Coomassie Brilliant Blue R-250 dye. No more protein spots appeared when the gel was stained with the more sensitive Silver stain (data not shown). The proteins could be classified according to the differences in their distribution (*Mr* and pI) in the 2DE-gel into five groups (**Figure 53**, lower panel): the first group was a protein of *Mr* ~50 kDa with a low pI (circle no. 1); the second group consisted of components of ~50 to 116 kDa with a high pI (circles no. 2-12); the third group was proteins of *Mr* ~25 to 35 kDa with a high pI (circles no. 13-15); the fourth group was proteins of *Mr* 7 to 20 kDa with a relatively broad pI range from ~3-7 (circles no. 16-21); and the fifth group included proteins of *Mr* ~7 to 20 kDa with a high pI (circles no. 22-24). **Table 9** shows orthologous proteins in the database matched with the proteins in circles no. 1-7, 11, 16-21, and 23-24 of *N. kaouthia* venom separated by 2DE at non-linear pH 3-10. Orthologs are not available in the database for the proteins in circles 8, 9, 10, 12-15 and 22. Proteins in circles no. 1 and 3-6 were identified as cobra venom factor. Matched peptides of protein in circle no. 1 are located within the β-chain (amino acids 1,264-1,642) of cobra venom factor (accession no. I51018) while matched peptides of proteins in circles no. 3-6 are located within the α-chain (amino acids 11-649) of cobra venom factor (**Figure 54**). Proteins in circles no. 16-21, which have different pI with slightly different *Mr* (lower panel, **Figure 53**), were identified as phopholipase A2 (accession numbers: P15445, P25498, PSNJ2K, 1OWSA) and the locations of their matched peptides are shown (**Figure 55**). Orthologous proteins in database matched with the tryptic peptide sequences from the study proteins are presented in **Appendix K Supplemental Table 1**.

Appendix L contains examples of peptide mass maps from 2DE-LC/MS-MS of cobra venom proteins in circles 1, 3-6 and 16-21.

3.3 Proteome of *N. kaouthia* venom identified by two dimensional (2D)-LC/MS-MS

A total of 61 orthologous proteins in the database matched with the peptide sequences derived from the peptide spectra of individual venom components generated by the 2D-LC/MS-MS. The orthologs of the *N. kaouthia* venom components could be classified into 12 different groups according to their putative biological functions/activities (**Appendix M**). These are: cardiotoxins, cobra venom factors, a cysteine-rich venom toxin, cytotoxins, kaouthiagin, mocarhagin, muscarinic toxin-like proteins, neurotoxins, an oxoglutarate dehydrogenase complex, phospholipases, serum albumin, and a weak toxin. **Appendix N** shows mass spectra and peptide sequences of the oxoglutarate dehydrogenase complex and the serum albumin.

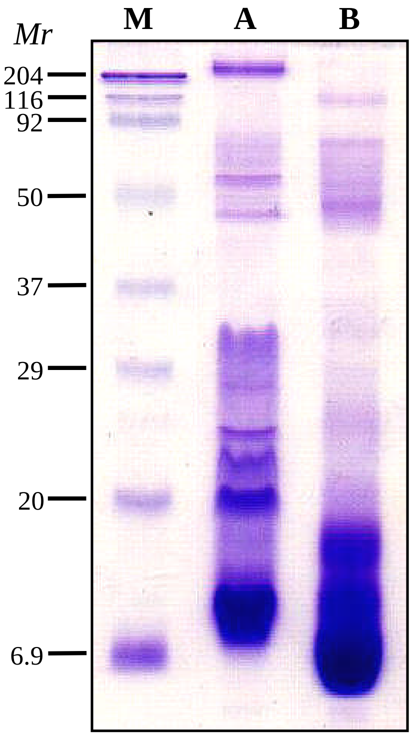


Figure 52 SDS-PAGE-patterns of *N. kaouthia* venom.

Fifteen µg of the venom was separated in a 15% polyacrylamide gel under non-reducing condition (lane A) and reducing condition (lane B)

Lane M is the Prestained Broad Range standard

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

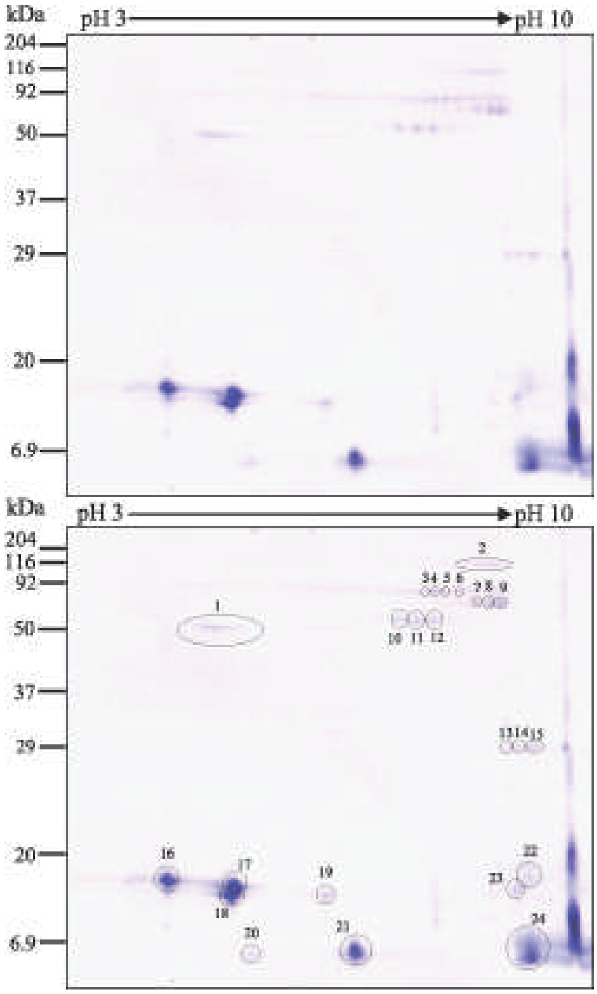


Figure 53 Proteomic pattern of *N. kaouthia* venom.

Six hundred micrograms of the venom after two dimensional gel electrophoresis at non-linear pH 3-10, stained by Coomassie Brilliant Blue R-250 dye.

Encircled and numbered proteins in the lower panel were subjected to LC/MS-MS and cross-referenced for orthologs in the database.

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

Table 9 Orthologous proteins of the database matched with the peptide sequences generated from tryptic digestion of proteins in circles no. 1-7, 11, 16-22 and 24 of *N. kaouthia* venom components separated by 2DE at non-linear pH 3-10 (lower panel, **Figure 53**). Orthologs are not available for proteins in circles no. 8-10, 12-15, and 23.

Circle no.	Orthologous protein(s)	Accession no.	Matched peptides	Top X_{corr} (charge)
1	Cobra venom factor precursor	I51018	10	5.692 (+2)
	K.LNQDITVTASGDGK.A K.ACETNVDYVYK.T R.WPHEDECQEEEFQK.L K.VYSYYNLDEK.C K.DKISYIITK.N K.ISYIITK.N R.IDVPLQIEK.A R.INYENALLAR.T K.VNDDYLIWGSR.S K.ATMTILTFYNAQLQEK.A			
	Complement C3 precursor	Q01833	4	4.193 (+2)
	R.KCQEALNLK.L K.VSHSEDECLHFK.I K.CQEALNLK.L K.HFEVGFIQPGSVK.V			
2	Nerve growth factor β-chain precursor	A59218	1	2.487 (+2)
	R.FIRIETACVCVITK.K			
3	Cobra venom factor precursor	I51018	4	5.834 (+2)
	K.GDNLIQMPGAAMK.I R.KYVLPSFEVR.L R.VDMNPAGGMLVTPTIEIPAK.E K.QLDIFVHDFPR.K			
	Complement C3 precursor	Q01833	3	5.218 (+2)
	R.TDTEEQILVEAHGDSTPK.S R.EYVLPSFEVR.L K.YFTYLILNK.G			
4	Cobra venom factor precursor	I51018	5	5.688 (+2)
	R.VFSMDHNTSK.M K.GDNLIQMPGAAMK.I R.QNQYVVVQVTGPQVR.L R.VDMNPAGGMLVTPTIEIPAK.E R.KLVLPSFEVR.L			

X_{corr}, cross correlation score; C3, complement component 3

Table 9 (continued)

Circle no.	Orthologous protein(s)	Accession no.	Matched peptides	Top X_{corr} (charge)
5	Cobra venom factor precursor	I51018	4	5.821 (+2)
	K.GDNLIQMPGAAMK.I R.QNQYVVVQVTGPQVR.L R.VDMNPAGGMLVTPTIEIPAK.E R.KYVLPSFEVR.L			
	Complement C3 precursor	Q01833	3	4.157 (+3)
	K.IKLEGDPGAR.V R.TDTEEQILVEAHGDSTPK.S R.EYVLPSFEVR.L			
6	Cobra venom factor precursor	I51018	4	6.010 (+2)
	K.GDNLIQMPGAAMK.I R.QNQYVVVQVTGPQVR.L R.KYVLPSFEVR.L R.VDMNPAGGMLVTPTIEIPAK.E			
	Complement C3 precursor	Q01833	3	3.669 (+2)
	R.TDTEEQILVEAHGDSTPK.S R.EYVLPSFEVR.L K.YFTYLILNK.G			
7	Chain A structure of an acidic phospholipase A2 of *Ophiophagus Hannah*	1M8TA	1	2.167 (+2)
	K.ENINDTTTR.C			
8, 9, 10	No matched protein in the database	-	-	-
11	Venom protein 2	NP508025.1	1	2.404 (+2)
	K.YGSEVESDIAAILEVFSKLR.Y			
12, 13, 14, 15	No matched protein in the database	-	-	-
16	PA2_NAJNA phospholipase A2, acidic phosphatidylcholine	P15445	4	5.327 (+2)
	R.GGSGTPVDDLDR.C K.TYSYECSQGTLTCK.G K.NMIKCTVPSR.S R.CCQVHDNCYNEAEK.I			

X_{corr}, cross correlation score

Table 9 (continued)

Circle no.	Orthologous protein(s)	Accession no.	Matched peptides	Top X_{corr} (charge)
16	Phospholipase A2 (EC 3.1.1.4) II R.CCQVHDNCYDEAEK.I K.NMIQCTVPNR.S NLYQFKNMIQCTVPNR.S	PSNJ2K	3	5.158 (+2)
	Chain A of C49 phospholipase A2 of Indian cobra (*N. naja sagittifera*) K.GNNNACAASSCDCDR.L R.NMIECTVPSR.S K.TYTYQCSGGTLTCK	1OWS	3	3.101 (+2)
17	Phospholipase A2 (EC 3.1.1.4.) II R.CCQVHDNCYDEAEK.I K.NMIQCTVPNR.S R.GGSGTPVDDLDRCCQVHDNCYDEAEKI R.LAAICFAGAPYNNNNYNIDLK.A R.CWPYFK.T	PSNJ2K	5	5.194 (+3)
	Chain A of a C49 phospholipase A2 of Indian cobra R.NMIECTVPSR.S K.TYTYQCSGGTLTCK.G	1OWS	2	3.177 (+2)
	PA2_NAJNA phopholipase A2, acidic phosphatidylcholine K.TYSYECSQGTKTCK.G R.GGSGTPVDDLDR.C K.ISGCWPYFK.T R.SWWDFADYGCYCGR.G	P15445	4	4.975 (+2)
	PA21_NAJOX phospholipase A2 R.LAAICFAGAPYNNDNYNINLK.A R.SWLDFANYGCYCGR.G	P25498	2	5.119 (+2)
	Chain B of phospholipase A2 of Indian R.CCQTHDNCYNEAEK.I	1MH2B	1	3.740 (+3)
18	PA2_NAJNA phospholipase A2 R.CCQVHDNCYNEAEK.I K.TYSYECSQGTLTCK.G K.NMIKCTVPSR.S R.GGSGTPVDDLDR.C	P15445	4	4.959 (+2)

X_{corr}, cross correlation score

Table 9 (continued)

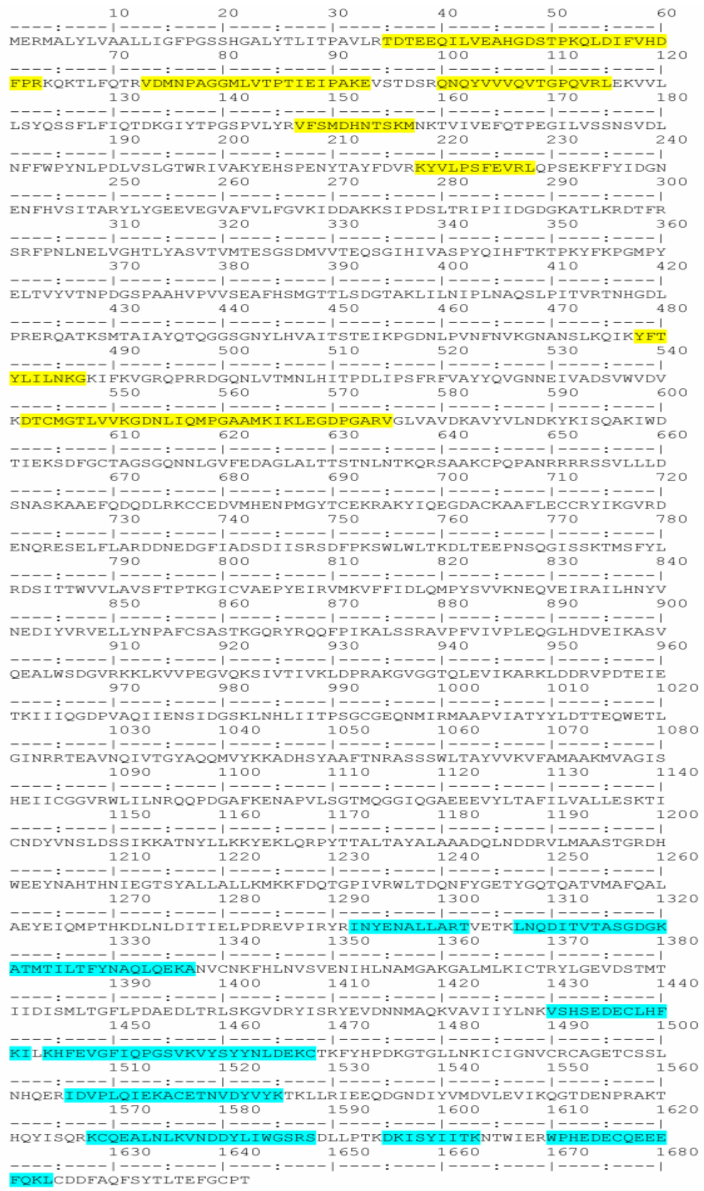
Circle no.	Orthologous protein(s)	Accession no.	Matched peptides	Top X_{corr} (charge)
18	Phospholipase A2 (EC 3.1.1.4.) II R.CCQVHDNCYDEAEK.I R.GGSGTPCDDLDRCCQVHDNCYDEAEK.I K.NMIQCTVPNR.S	PSNJ2K	3	5.282 (+3)
	PA21_NAJAT phospholipase A2 precursor (phosphatidylcholine) K.GGNNACAAAVCDCDR.L K.NMIQCTVPSR.S	P00598	2	4.105 (+2)
19	Chain A of a C49 phospholipase A2 of Indian cobra K.TYTYQCSGGTLTCK.G R.LAAICFAGAPYNDNNYNIDLK.A	1OWSA	2	6.173 (+3)
	PA2_NAJNA phopholipase A2, acidic phosphatidylcholine R.GGSGTPVDDLDR.C K.TYSYECSQGTLTCK.G K.ISGCWPYFK.T R.SWWDFADYGCYCGR.G	P15445	4	5.466 (+3)
19	Phospholipase A2 (EC 3.1.1.4.) II R.CCQVHDNCYDEAEK.I K.NMIQCTVPNR.S R.LAAICFAGAPYNNNNYNIDLK.A	PSNJ2K	3	5.654 (+2)
20	PA21_NAJOX phospholipase A2 isozyme E (phosphatidylcholine) R.LAAICFAGAPYNNDNYNINLK.A R.SWLDFANYGCYCGR.G	P25498	2	4.885 (+2)
	Chain A of A C49 phospholipase A2 of Indian cobra R.LAAICFAGAPYNNDNYNINLK.A	1OWS	1	5.925 (+3)
21	PA21_NAJOX phospholipase A2 isozyme E (phosphatidylcholine) R.LAAICFAGAPYNNDNYNINLK.A R.SWLDFANYGCYCGR.G	P25498	2	5.104 (+2)

X_{corr}, cross correlation score

Table 9 (continued)

Circle no.	Orthologous protein(s)	Accession no.	Matched peptides	Top X_{corr} (charge)
21	Chain A, crystal structure of A C49 phospholipase A2	10WS	1	5.742 (+2)
	R.LAAICFAGAPYNDNNYNIDLK.A			
	PA2_NAJNA phospholipase A2, acidic (phosphatidylcholine)	P15445	2	5.202 (+2)
	K.ISGCWPYFK.T			
	R.SWWDFADYGCYCGR.G			
22	Cytotoxin 1 of monocled cobra	H3NJ1K	1	3.117 (+2)
	K.MFMMSDLTIPVK.R			
23	No matched protein in the database	-	-	-
24	NXL1_NAJKA long neurotoxin 1 (neurotoxin 3 or α–cobra toxin)	P01391	3	5.808 (+2)
	K.TGVDIQCCSTDNCNPFPTR.K			
	R.CFITPDITSK.D			
	K.TWCDAFCSIR.G			
	NXL2_NAJNA long neurotoxin 2 (toxin B)	P25669	1	5.595 (+3)
	R.TGVDIQCCSTDDCDPFPTRK.R			

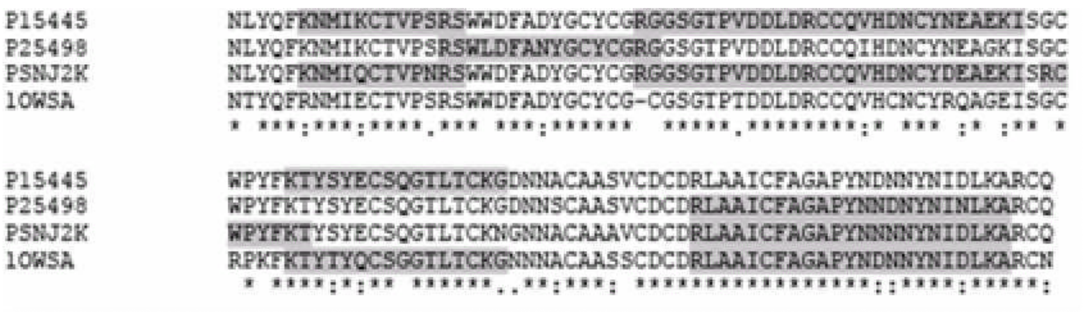
X_{corr}, cross correlation score



```
        10        20        30        40        50        60
----:----|----:----|----:----|----:----|----:----|----:----|
MERMALYLVAALLIGFPGSSHGALYTLITPAVLRTDTEEQILVEAHGDSTPKQLDIFVHD
        70        80        90        100       110       120
----:----|----:----|----:----|----:----|----:----|----:----|
FPRKQKTLFQTRVDMNPAGGMLVTPTIEIPAKEVSTDSRQNQYVVVQVTGPQVRLEKVVL
        130       140       150       160       170       180
----:----|----:----|----:----|----:----|----:----|----:----|
LSYQSSFLFIQTDKGIYTPGSPVLYRVFSMDHNTSKMNKTVIVEFQTPEGILVSSNSVDL
        190       200       210       220       230       240
----:----|----:----|----:----|----:----|----:----|----:----|
NFFWPYNLPDLVSLGTWRIVAKYEHSPENYTAYFDVRKYVLPSFEVRLQPSEKFFYIDGN
        250       260       270       280       290       300
----:----|----:----|----:----|----:----|----:----|----:----|
ENFHVSITARYLYGEEVEGVAFVLFGVKIDDAKKSIPDSLTRIPIIDGDGKATLKRDTFR
        310       320       330       340       350       360
----:----|----:----|----:----|----:----|----:----|----:----|
SRFPNLNELVGHTLYASVTVMTESGSDMVVTEQSGIHIVASPYQIHFTKTPKYFKPGMPY
        370       380       390       400       410       420
----:----|----:----|----:----|----:----|----:----|----:----|
ELTVYVTNPDGSPAAHVPVVSEAFHSMGTTLSDGTAKLILNIPLNAQSLPITVRTNHGDL
        430       440       450       460       470       480
----:----|----:----|----:----|----:----|----:----|----:----|
PRERQATKSMTAIAYQTQGGSGNYLHVAITSTEIKPGDNLPVNFNVKGNANSLKQIKYFT
        490       500       510       520       530       540
----:----|----:----|----:----|----:----|----:----|----:----|
YLILNKGKIFKVGRQPRRDGQNLVTMNLHITPDLIPSFRFVAYYQVGNNEIVADSVWVDV
        550       560       570       580       590       600
----:----|----:----|----:----|----:----|----:----|----:----|
KDTCMGTLVVKGDNLIQMPGAAMKIKLEGDPGARVGLVAVDKAVYVLNDKYKISQAKIWD
        610       620       630       640       650       660
----:----|----:----|----:----|----:----|----:----|----:----|
TIEKSDFGCTAGSGQNNLGVFEDAGLALTTSTNLNTKQRSAAKCPQPANRRRRSSVLLLD
        670       680       690       700       710       720
----:----|----:----|----:----|----:----|----:----|----:----|
SNASKAAEFQDQDLRKCCEDVMHENPMGYTCEKRAKYIQEGDACKAAFLECCRYIKGVRD
        730       740       750       760       770       780
----:----|----:----|----:----|----:----|----:----|----:----|
ENQRESELFLARDDNEDGFIADSDIISRSDFPKSWLWLTKDLTEEPNSQGISSKTMSFYL
        790       800       810       820       830       840
----:----|----:----|----:----|----:----|----:----|----:----|
RDSITTWVVLAVSFTPTKGICVAEPYEIRVMKVFFIDLQMPYSVVKNEQVEIRAILHNYV
        850       860       870       880       890       900
----:----|----:----|----:----|----:----|----:----|----:----|
NEDIYVRVELLYNPAFCSASTKGQRYRQQFPIKALSSRAVPFVIVPLEQGLHDVEIKASV
        910       920       930       940       950       960
----:----|----:----|----:----|----:----|----:----|----:----|
QEALWSDGVRKKLKVVPEGVQKSIVTIVKLDPRAKGVGGTQLEVIKARKLDDRVPDTEIE
        970       980       990       1000      1010      1020
----:----|----:----|----:----|----:----|----:----|----:----|
TKIIIQGDPVAQIIENSIDGSKLNHLIITPSGCGEQNMIRMAAPVIATYYLDTTEQWETL
        1030      1040      1050      1060      1070      1080
----:----|----:----|----:----|----:----|----:----|----:----|
GINRRTEAVNQIVTGYAQQMVYKKADHSYAAFTNRASSSWLTAYVVKVFAMAAKMVAGIS
        1090      1100      1110      1120      1130      1140
----:----|----:----|----:----|----:----|----:----|----:----|
HEIICGGVRWLILNRQQPDGAFKENAPVLSGTMQGGIQGAEEEVYLTAFILVALLESKTI
        1150      1160      1170      1180      1190      1200
----:----|----:----|----:----|----:----|----:----|----:----|
CNDYVNSLDSSIKKATNYLLKKYEKLQRPYTTALTAYALAAADQLNDDRVLMAASTGRDH
        1210      1220      1230      1240      1250      1260
----:----|----:----|----:----|----:----|----:----|----:----|
WEEYNAHTHNIEGTSYALLALLKMKKFDQTGPIVRWLTDQNFYGETYGQTQATVMAFQAL
        1270      1280      1290      1300      1310      1320
----:----|----:----|----:----|----:----|----:----|----:----|
AEYEIQMPTHKDLNLDITIELPDREVPIRYRINYENALLARTVETKLNQDITVTASGDGK
        1330      1340      1350      1360      1370      1380
----:----|----:----|----:----|----:----|----:----|----:----|
ATMTILTFYNAQLQEKANVCNKFHLNVSVENIHLNAMGAKGALMLKICTRYLGEVDSTMT
        1390      1400      1410      1420      1430      1440
----:----|----:----|----:----|----:----|----:----|----:----|
IIDISMLTGFLPDAEDLTRLSKGVDRYISRYEVDNNMAQKVAVIIYLNKVSHSEDECLHF
        1450      1460      1470      1480      1490      1500
----:----|----:----|----:----|----:----|----:----|----:----|
KILKHFEVGFIQPGSVKVYSYYNLDEKCTKFYHPDKGTGLLNKICIGNVCRCAGETCSSL
        1510      1520      1530      1540      1550      1560
----:----|----:----|----:----|----:----|----:----|----:----|
NHQERIDVPLQIEKACETNVDYVYKTKLLRIEEQDGNDIYVMDVLEVIKQGTDENPRAKT
        1570      1580      1590      1600      1610      1620
----:----|----:----|----:----|----:----|----:----|----:----|
HQYISQRKCQEALNLKVNDDYLIWGSRSDLLPTKDKISYIITKNTWIERWPHEDECQEEE
        1630      1640      1650      1660      1670      1680
----:----|----:----|----:----|----:----|----:----|----:----|
FQKLCDDFAQFSYTLTEFGCPT
```

Figure 54 Locations of tryptic peptides generated from proteins in circles no. 1 and no. 3-6 within cobra venom factor (accession no. I51018).

Matched tryptic peptides generated from protein in circle no. 1 (yellow shade) are located within the β-chain of the cobra venom factor (amino acids 1,264-1,642). Matched tryptic peptides generated from proteins in circles no. 3-6 (blue shade) are located within the α-chain (amino acids 11-649).



```
P15445          NLYQFKNMIKCTVPSRSWWDFADYGCYCGRGGSGTPVDDLDRCCQVHDNCYNEAEKISGC
P25498          NLYQFKNMIKCTVPSRSWLDFANYGCYCGRGGSGTPVDDLDRCCQIHDNCYNEAGKISGC
PSNJ2K          NLYQFKNMIQCTVPNRSWWDFADYGCYCGRGGSGTPVDDLDRCCQVHDNCYDEAEKISRC
1OWSA           NTYQFRNMIECTVPSRSWWDFADYGCYCG-CGSGTPTDDLDRCCQVHCNCYRQAGEISGC
                * ***:***:****.*** ***:******  *****.********:* *** :* :** *

P15445          WPYFKTYSYECSQGTLTCKGDNNACAASVCDCDRLAAICFAGAPYNDNNYNIDLKARCQ
P25498          WPYFKTYSYECSQGTLTCKGDNNSCAASVCDCDRLAAICFAGAPYNNDNYNINLKARCQ
PSNJ2K          WPYFKTYSYECSQGTLTCKNGNNACAAAVCDCDRLAAICFAGAPYNNNNYNIDLKARCQ
1OWSA           RPKFKTYTYQCSGGTLTCKGNNNACAASSCDCDRLAAICFAGAPYNDNNYNIDLKARCN
                 * ****:*:** ******..**:***: ****************::****:*****:
```

Figure 55 Locations of tryptic peptides generated from proteins in circles no. 16-21 within phospholipase A2 (accession no. P15445, P25498, PSNJ2K and 1OWSA).

Matched tryptic peptides generated from proteins in circles no. 16 and 18 are located within phospholipase (accession no. P15445).

Matched tryptic peptides generated from protein in circle no. 17 are located within phospholipase A2 (accession no. PSNJ2K).

Matched tryptic peptides generated from protein in circle no. 19 are located within phospholipase A2 (accession no. 1OWSA).

Matched tryptic peptides generated from protein in circles no. 20 and 21 are located within phospholipase A2 (accession no. 25498).

Multiple alignment program was performed by ClustalW 1.83 (http://bioweb.pasteur.fr/seqanal/interfaces/emma-simple.html).

Identical amino acids are indicated by (*). Similar chemical properties of unidentical amino acid side chain are indicated by (**.**) or (**:**).

4. Fractionation of protein components of *N. kaouthia* venom

The protein components of the venom were separated by using ion exchange column chromatography followed the method of Karlsson *et al*. (1971) with some modifications. The total volume of the gradients of 0.14 to 1.2 M ammonium acetate pH 6.5 was 600 ml. The graph as shown in **Figure 56** was plotted; X axis was fraction numbers, Y axis on left hand side was OD at A_{280nm}, and Y axis on right hand side was the concentration of ammonium acetate. The venom protein components were separated into 11 peaks (**Figure 56**). Peaks numbered 1 to 5 (P1-P5) were unbound proteins eluted by 0.09 M ammonium acetate pH 6.5. Peaks numbered 6-11 (P6-P11) were the CM-bound proteins that were eluted by using gradients of ammonium acetate pH 6.5 from 0.14 M to 1.2 M. Finally, any proteins that sill retained in the column were eluted by using 1.4 M ammonium acetate pH 6.5 (designated P12).

Protein fractions in each peak from the graph were pooled and concentrated. The ammonium acetate buffer, that venom protein was dissolved, was changed to DW by using Amicon® Ultra-15 Centrifugal Filter Device (Milli® Pore, MA, USA) with molecular weight cut-off at 5 kDa. The protein(s) in each peak was/were analyzed on 15% SDS-PAGE under either non-reducing condition (**Figure 57A**) or reducing condition (**Figure 57B**) and staining with Coomassie™ Brilliant Blue G-250.

The protein with high molecular mass at > 204 kDa in lane 1 of **Figure 57A** was eluted at the first peak of the unbound proteins (**P1** in **Figure 57**). The unbound proteins in lanes 3 and 5 showed similar pattern at molecular mass about 20 kDa, while those of lanes 2 and 4 had molecular masses about 6.9 and 10 kDa, respectively. After all of the unbound proteins had been washed, six protein peaks (P6-P11) were eluted with ammonium acetate. They showed different banding patterns on SDS-PAGE. Lanes no. 8-11 of **Figure 57** show single band of protein but lanes no. 10 and 11 of **Figure 57** showed smear bands of proteins that might be glycoproteins. After the concentration of the ammonium acetate salt reached 1.2 M, there was no protein eluted out. Nevertheless, all remaining proteins could be eluted at 1.4 M of the salt (lane 12**, Figure 57**).

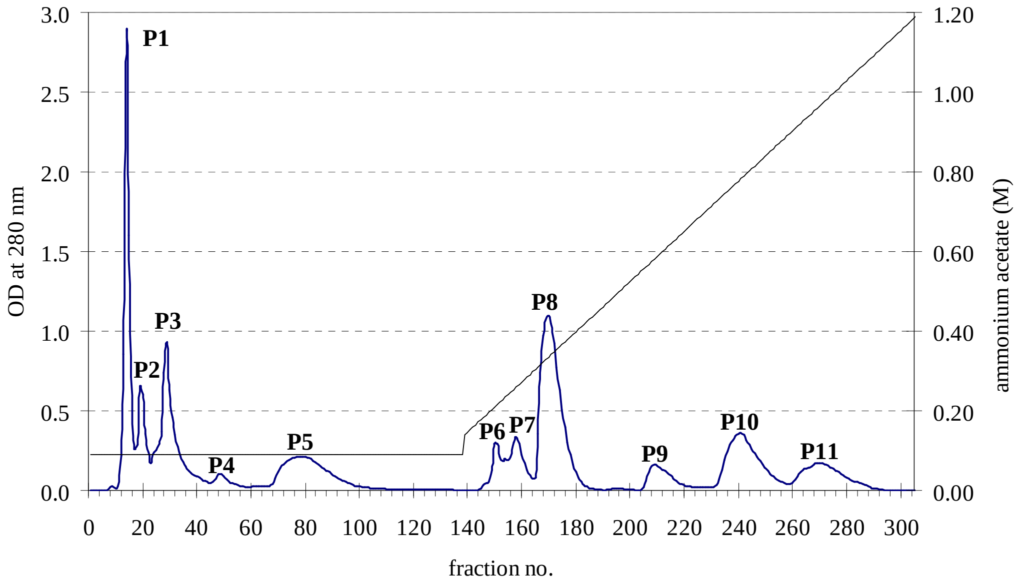


Figure 56 Elution profiles of crude *N. kaouthia* venom from ion exchange column chromatography.

P1-P5, the unbound proteins eluted by 0.09 M ammonium acetate, pH 6.5.

P6-P11, the CM-bound proteins eluted by gradients of ammonium acetate, pH 6.5 from 0.14 M to 1.2 M.

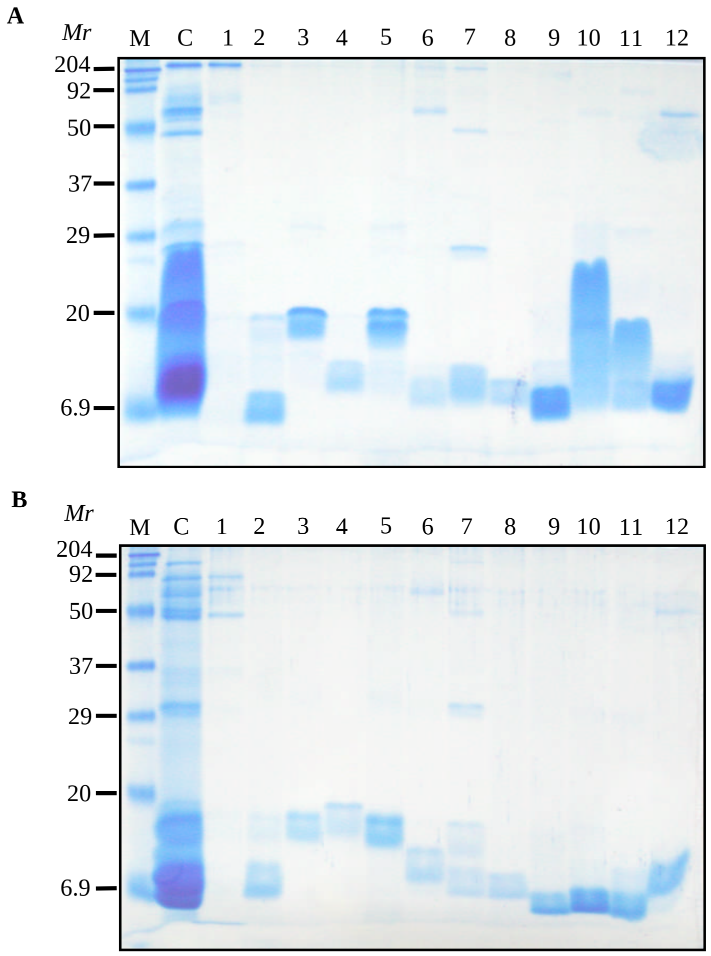


Figure 57 SDS-PAGE patterns of proteins in peaks numbered 1 to 11 (**P1-P11 of Figure 56**) analyzed on 15% SDS-PAGE and stained with Coomassie™ Brilliant Blue G-250 under either non-reducing condition (**A**) or reducing condition (**B**).

Lanes M, the prestained broad range protein standard

Lanes C, the crude *N. kaouthia* venom

Lanes 1-5, the unbound proteins in P1-P5, respectively

Lanes 6-11, the bound proteins in P6-P11, respectively

Lanes 12, the 1.4 M ammonium acetate-eluted proteins

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

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

The median lethal dose (LD50) by means of the intra-peritoneal route (IP) was determined following WHO protocol. **Table 10** shows the numbers of alive and dead mice after 24 hours of intra-peritoneal injection of crude *N. kaouthia*. The average LD50 of crude *N. kaouthia* calculated by the method of Reed and Muench (1938) was 4.36 μg per mouse.

For intra-muscular route, **Table 11** shows the numbers of alive and dead mice at 24 hours after the intra-muscular injection of crude *N. kaouthia*. The average LD50 of crude venom calculated by the method of Reed and Muench (1938) was 4.86 μg per mouse.

6. Lethal activity of venom protein components

The column-separated venom protein components (P1-P11 in **Figures 56** and **57**) at 2.18 μg (0.5x LD50, IP) was injected into intra-peritoneal cavities of two male ICR mice. **Table 12** shows the numbers of alive and dead mice at 24 hours after the venom protein injection. Proteins in P4 and P8 contained lethal toxin(s) that caused death of both mice, in the groups. The proteins in P4 and P8 were subsequently identified by 2D-LC/MS-MS (please see **Section 3.3** of **Chapter IV**).

7. Identification of the column-separated venom protein components

The column-separated venom protein components (**P1, P3, P4, P5,** and **P8** of **Figure 57**) were identified by using mass spectrometry and database search. The band of protein at *Mr* >204 kDa (**Figure 57, lane 1**) of fraction P1 was excised out from the 15% polyacrylamide gel and subjected to the matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) which was performed at the BSU, BIOTECH, NSTDA, Thailand. The total number of peptide queries was 51 and only 40 peptide queries were matched with peptides of the database. **Figure 58** shows the mass spectrum of the trypsin-digested peptides. The details of the 40 trypsin-digested peptide sequences generated by the MALDI-TOF are shown in **Appendix O**.

Table 10 Numbers of alive and dead mice at 24 hours after intra-peritoneal injection with various amounts of crude *N. kaouthia* venom

Triplicate experiment no.	Amount of crude venom proteins (μg)										LD50* (μg/mouse)
	1.0		2.0		3.0		4.0		5.0		
	A	D	A	D	A	D	A	D	A	D	
1	5	0	5	0	5	0	4	1	1	4	4.47
2	5	0	5	0	5	0	3	2	0	5	4.15
3	5	0	5	0	5	0	3	2	2	3	4.47
										Mean	4.36

A = alive; D = dead

*calculated by the method of Reed and Muench, 1938

Table 11 Number of alive and dead mice at 24 houes after intra-muscular injection of various amounts of crude *N. kaouthia*.

Triplicate experiment no.	Amount of crude venom proteins (μg)										LD50* (μg/mouse)
	3.0		3.5		4.0		4.5		5.0		
	A	D	A	D	A	D	A	D	A	D	
1	5	0	4	1	5	0	2	3	4	1	4.80
2	5	0	1	4	4	1	5	0	4	1	4.78
3	5	0	5	0	5	0	4	1	3	2	4.99
										Mean	4.86

A = alive; D = dead

*calculated by the method of Reed and Muench, 1938

Table 12 Numbers of alive and dead mice at 24 hours after intra-peritoneal injection with 2.18 µg of column-fractionated crude *N. kaouthia* venom, P1 to P11 (0.5x LD50 by means of intra-peritoneal route)**.**

Mouse condition	P1	P2	P3	P4*	P5	P6	P7	P8*	P9	P10	P11
Alive	2	2	2	0	2	1	1	0	2	2	2
Dead	0	0	0	2	0	1	1	2	0	0	0

*Highly lethal fraction; *Mr* ~7-10 kDa

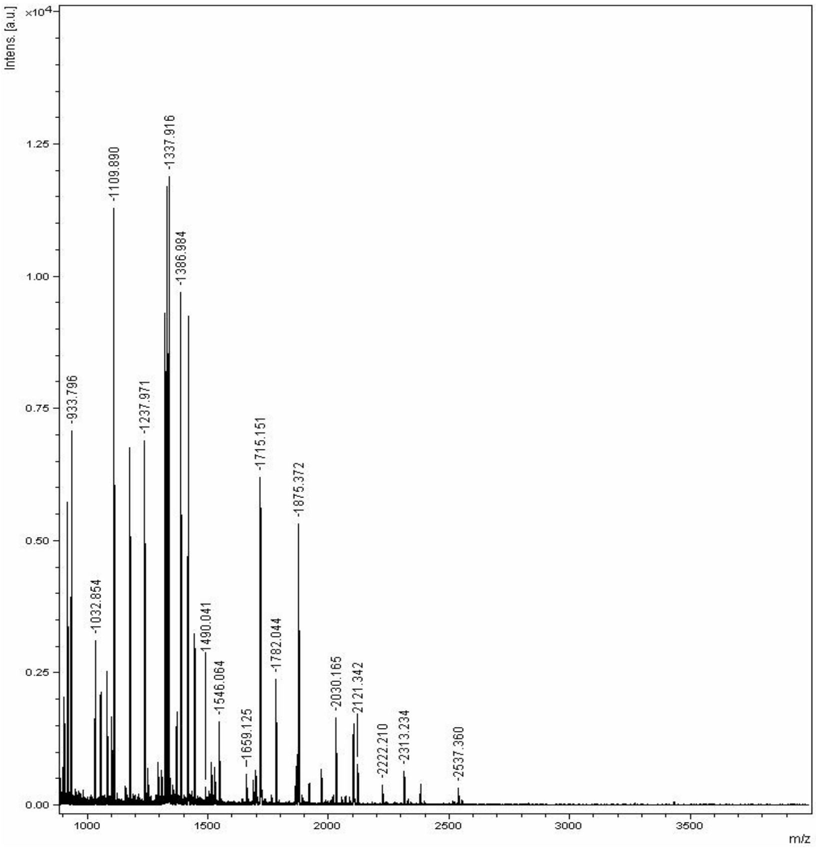


Figure 58 Mass spectrum of the P1 proteins (**Figure 57A**) which was identified as cobra venom factor by MALDI-TOF (accession no. 54035743)

8. Identification of protein components by 2D-LC/MS-MS

Protein components in P3, P4, P5, and P8 were identified by 2D-LC/MS-MS as described in **Section 4.4** of **Chapter IV**. **Table 13** contains the results of the 2D-LC/MS-MS identification of the four protein peaks. The top ten of orthologous proteins which gave high score matching with proteins in the fractions are shown and the details of the tryptic peptides are given in Appendix P.

In conclusion, the P3 contained predominantly phospholipase isoenzymes, and cobra venom factor; the P4 contained natrin, albumin, Thai cobrin, and cobra venom factor; the P5 contained predominantly phospholipase isoenzymes, and cobra venom factor, and the P8 contained principally long neurotoxin, natrin, and toxin B (**Table 13**)**.**

Table 13 Orthologs of the venom proteins in P3, P4, P5, and P8 identified by 2D-LC/MS-MS after orthologous search

P3	**P4**	**P5**	**P8**
1. CO3_NAJKA cobra venom factor precursor (CVF)	1. CO3_NAJKA cobra venom factor precursor (CVF)	1. CO3_NAJKA cobra venom factor precursor (CVF)	1. NXL1_NAJKA long neurotoxin 1 (Neurotoxin 3) (α-cobratoxin)
2. PSNJ2K phospholipase A2 (EC 3.1.1.4)	2. Albumin [*Bos taurus*]	2. PA21_NAJKA phospholipase A2	2. NXL3_NAJNA long neurotoxin 3 (Toxin C)
3. PA21_NAJAT phospholipase A2 1 precursor	3. ALBU_BOVIN serum albumin precursor	3. PSNJ2K phospholipase A2 (EC 3.1.1.4) II	3. A crystal structure of natrin from *Naja Atra* venom
4. Phospholipase A2 (E.C.3.1.1.4)	4. 754920A Albumin	4. PA21_NAJAT phospholipase A2	4. A crystal structure of natrin from *Naja atra*
5. PA22_NAJAT phospholipase A2	5. A crystal structure of natrin from *Naja atra*	5. CO3_NAJNA complement C3 precursor	5. Toxin B
6. PSNJ3K phospholipase A2 (EC 3.1.1.4)	6. A crystal structure of natrin from Taiwan Cobra (*Naja atra*)	6. Phospholipase A2 (E.C.3.1.1.4)	6. NXL4_NAJNA Long neurotoxin 4 (Toxin D)
7. PA21_NAJKA phospholipase A2	7. ALB protein (*Bos taurus*)	7. Natrin from *Naja atra* snake venom	7. NXL5_NAJNA long neurotoxin 5 (Toxin E)

Table 13 (Contined.).

P3	P4	P5	P8
8. CO3_NAJNA complement C3 precursor	8. THAI_NAJKA Thai cobrin	8. PA2_NAJNA phospholipase A2	8. CRVP1_NAJAT natrin-1 precursor (cysteine-rich venom protein 1)
9. PA2_NAJNA phospholipase A2, acidic (Phosphatidylcholine 2-acylhydrolase)	9. CRVP1_NAJAT natrin-1 precursor (cysteine-rich venom protein 1)	9. PSNJ3K phospholipase A2 (EC 3.1.1.4) III - monocled cobra	9. NXL1_NAJNA long neurotoxin 1 (Toxin A)
10. PA2D_NAJSP phospholipase A2, acidic D precursor (Phosphatidylcholine 2-acylhydrolase) (APLA)	10. Serum albumin (*Bos indicus*)	10. PA2D_NAJSP phospholipase A2, acidic D precursor (Phosphatidylcholine 2-acylhydrolase) (APLA)	10. NXL1_NAJNA long neurotoxin 1 (Toxin A)