CHAPTER VI
DISCUSSION

1. Human antibody phage display library

In this study, a human antibody phage display library, *i.e.*, a single pot library was constructed. The human antibodies in the form of single chain variable fragments (HuScFv) were displayed on surface of filamentous M13 phages as a fusion partner to the surface coat pIII protein of the phages while the respective *huscFv* gene sequences were molecularly linked to g3p of the phages in the phage genome. The pCANTAB5E phagemid vector system and a helper phage, *i.e.*, M13KO7 were used in the phage library construction. This phagemid system was selected because of the smaller size of the *huscFv*-pCANTAB5E comparred with the phage genome and it was easier than the phage genome in the gene insertion and the bacterial transformation. By using the pCANTAB5E, however, not all of the pIII molecules on the tip of each phage particle were displaying the HuScFv but some of them are free. This allows the phage to infect into *E. coli* host cell by using the pIII as the phage ligand and the F pilus of the bacteria as the receptor.

Human immunoglobulin genes were obtained from peripheral blood mononuclear cells (PBMC) of young adult 50 Thai volunteers and 10 buffy coat samples of the donated bloods from the Thai Blood Bank. The PBMC had more than 95% viability when the total RNA was extracted from each preparation. A total volume of the whole venous blood used for PBMC isolation was ~3,750 ml (25 ml of whole venous blood of 50 volunteers and 250 ml of donated whole venous blood of blood donors). The blood volume and a multiplicity of the blood donors were larger than the naïve human antibody phage library constructed by Nissim *et al.* (1994) and Vaughan *et al.* (1996). Each total RNA had good quality as shown by the presence of DNA bands of 28 S and 8 S DNA. Thus they were reversed transcribed to cDNA; all the cDNA preparations were pooled and used as templates for amplifications of gene sequences encoding variable heavy and light chains (VH and VL-coding sequences).

Multiple pairs of forward and reverse primers were used to amplify all families of immunoglobulin-coding sequences from the pooled cDNA. Pimers were designed

from multiple alignments of human functional immunoglobulin genes in VBASE. More than a thousand published sequences which had been deposited in the Genbank and EMBL data libraries were aligned. The database had been constructed at the MRC Centre for Protein Engineering (Cambridge, UK).

For *VH* amplifications, there were forty-two combinations of 14 forward and 3 reverse primers. The VH DNA amplicon (at ~450 bp) could be obtained from all primer combinations. By using equal volume of template, differences in the intensity of human *VH* amplicons were obtained which could depend on the degree of degeneracy of the primers. The primer with high degeneracy, *i.e., JH1245*, gave lower DNA band intensity than the primers *JH3* and *JH6* when they were individually combined with the 14 forward primers. The intensity of the *VH* band obtained by using the *JH1245* with the forward primers could be increased by using more amount of cDNA template.

Human *Vκ* were amplified using a total of 26 primer combinations. Among them, there are two primer combinations, *i.e., Vκ3c + Jκ5* and *Vκ5a + Jκ5* that gave no DNA amplicons and one primer combination, *i.e., Vκ1d + Jκ5* that gave low amount of the amplicon. These suggested that there were either small or nil of the copy numbers of the particular Vκ-encoding DNA sequences that its J gene segment contained *Jk5*.

The high efficiency of competent TG1 *E. coli* (~10^9 cfu/μg uncut pUC19) was used to construct the human antibody phage display library. After phage amplification, this library contained phages at 6.5 x 10^{12} cfu generated from the ~2.6 x 10^8 cfu of individual *huscFv*-phagemid-transformed TG1 *E. coli* cells.

2. Proteomics of *N. kaouthia* venom pool (a combination of venom milked from several snakes)

The protein components of the pool of the *N. kaouthia* venom from multiple snakes were revealed by proteomics. The *N. kaouthia* crude venom pool was first separated under the β-mercaptoethanol reduction by using 15% SDS-PAGE. The components of ~7 to 116 kDa were revealed which did not conform to the previous data by Byeon and Weisblum (Byeon and Weisbluum, 2004) who reported that the

venom components ranged in *Mr* from 6.5 to ~66 kDa. The difference was likely to reflect the difference in the geographical areas of the cobras where the venoms were obtained as previously documented (Wei *et al.*, 2003) and was less likely to be due to the seasonal variation; as published data on rattlesnakes showed no variation in the isoelectric focused patterns of the venom obtained from snakes kept under ambient temperatures that simulated seasonal changes (Gregory-Dwyer *et al.*, 1986).

In this study, we used 2DE-based- and 2D-LC/MS-MS to reveal the *N. kaouthia* venom proteome. Six hundred microgram aliquot of the venom pool was subjected to the sample preparation for the two dimensional-gel electrophoresis (2DE). However, the procedure used in sample preparation, *e.g.* precipitation using the 2D-Clean-Up kit and removal of DeStreak^{TM} solution-insoluble material for the 2DE, caused some loss of proteins. Thus, the pattern and intensity in the Coomassie Brilliant Blue stained-2DE-gel do not represent the 600 μg proteins of the crude venom pool. From the 2DE-proteomics, only 24 protein spots were revealed while up to 61 proteins were identified by 2D-LC/MS-MS. The difference is due to the limitation of the current gel-based (2DE-) proteomics. In the 2D-LC/MS-MS, the intact venom proteins dissolved in distilled water were directly digested by trypsin and all of the tryptic digested peptides were subjected to peptide mass map generation and database search.

Among the 16 proteins of the *N. kaouthia* venom derived from 2DE-LC/MS-MS for which orthologous proteins could be found in the database, many had different molecular masses and pI, but were identified as proteins of the same biological activity. For examples, the proteins in circles no. 1 (*Mr* ~50 kDa, pI 4-5) and no. 3-6 (*Mr* ~92 kDa, pI 8-9) were similarly identified as cobra venom factors. The proteins in circles no. 3-6 showed similar peptide mass fingerprints (**Appendix L**) and yet they had a different pI indicating that there might have been a post-translational modification of these abundant proteins (Hesketh *et al.*, 2002) with diverse biological functions. Further experiments are needed to pinpoint what kind of modification, if any, was involved. It is known that the cobra venom factor contains three subunits, *i.e.*, α-, β-, and γ-chains, with molecular masses of 70, 50, and 30 kDa, respectively, and that these subunits are linked by three inter-chain disulphide bonds (Osipov *et al.*, 2005b). Subunits α and β are glycosylated but data concerning glycosylation of the γ–chain are contradictory (Eggertsen *et al.*, 1981; Gowda *et al.*, 1992). Von Zabern *et*

al. (1982) found that under a 1-2 mM dithiothreitol reducing condition, cobra venom factor could be cleaved to yield free 50 kDa β-chains together with an intermediate product of disulphide-linked α-and γ-chains. In this study, prior to isoelectric-focusing in the first dimensional electrophoresis, the venom proteins were dissolved in a DeStreak™ Rehydration solution containing 10 mM dithiothreitol, which is a relatively strong reducing agent. We found that the protein in circle no. 1 had an *Mr* of ~50 kDa, and thus matched peptides were located within the β-chain of the cobra venom factor (accession no. I51018) while the proteins in circles no. 3-6 had an *Mr* of ~92 kDa and their matched peptides were located within the α-chain (accession no. I51018); thus it was possible that the proteins in spots no. 3-6 were disulfide linked α- and γ–chains, similar to the intermediate product described by von Zabern *et al.* (1982).

Many isoforms of phospholipase A_2 with a different *Mr* and pI have been revealed in this study by 2DE-based-LC/MS-MS. Our findings agree with data reported previously that cobra venom purified by conventional column chromatographies contained many isoforms of the phospholipase enzyme (Doley and Mukherjee, 2003; Doley *et al.*, 2004).

2DE-based proteomics has some limitations: the 2DE is not suitable for studying components in highly complex protein mixtures or membrane proteins of low solubility (Washburn *et al.*, 2001; Delahunty and Yates, 2005). Besides, currently available IPG strips for the first dimensional protein separation cannot be used for extremely acidic or basic proteins with a pI lower than 3 or higher than 12, respectively (Li *et al.*, 2004; Washburn *et al.*, 2001; Delahunty and Yates, 2005). It is not known whether the cobra venom contains any membrane proteins. Nevertheless, to overcome the limitations of the 2DE-based proteomics, we used 2D-LC/MS-MS for the separation and construction of peptide mass spectra of the venom components (Delahunty and Yates, 2005). We found that the 2D-LC/MS-MS could detect proteins of minute amounts in the mixture which were not revealed by the 2DE-LC/MS-MS. The former technique was used successfully for identification of membrane proteins and proteins with an extreme pI (Washburn *et al.*, 2001). By using the 2D-LC/MS-MS and database search, novel toxic and non-toxic protein components which have never been reported previously in the *N. kaouthia* venom were identified, such as the

components matched with the metalloproteinase mocarhagin found in *N. mossambica* ssp. *mossambica* and an oxoglutarate dehydrogenase complex found in *Burkholderia cenocepacia*. Moreover, a novel venom protein, natrin, was also identified by 2D-LC/MS-MS.

3. Production of human single chain antibody fragments (HuScFv) specific to venom protein components by using the own constructed phage display human antibody library

For production of the HuScFv specific to protein components of *N. kaouthia* venom, the protein components were separated into fractions by using a cationic exchange column chromatography followed the method of Karlsson *et al.* (1971) with some modifications. A total of 11 protein peaks, *i.e.*, P1-P11, were obtained from the chromatography of the crude venom pool. The P4, P6, P7, and P8 were lethal when injected into mice. The proteins in the P4 and P8 were identified as natrin and long α-neurotoxin, respectively, and they were used to select phage clones displaying HuScFv to the proteins by the bio-panning process. P1 and P3 and P5 which principally contained the cobra venom factor and phospholipases, respectively, were also used in the phage bio-panning to select HuScFv-phage clones. Karlsson *et al.* (1971) used the ion-exchange column chromatography to isolate only long α-neurotoxin; they also eluted the long α-neurotoxin out from column in the same peak (using the same molarity of acetate buffer) as in this study. Karlsson *et al*. (1971) tested the lethal, phospholipase, and coagulation activities of the toxin and their results showed that only lethal activity of this toxin fraction was observed which was similar to the result of this study. The different finding in lethal activity of individual components might reflect that the variation in venoms collected from cobras of different geographical areas as documented previously (Wei *et al.*, 2003).

Not only the protein peak that principally contained the long α-neurotoxin (P8) could caused death in mice, but also the P4 which principally contained the natrin. The observation agreed with those of Osipov *et al*. (2005) who found that natrin of the *N. haje* was lethal to mice.

HuScFv that could bind specifically to proteins in P1 (cobra venom factor), P4 (natrin), P3 and P5 (phospholipase A2), and P8 (long neurotoxin) of the crude venom pool could be successfully produced from *E. coli* clones that were transformed with the phage clones selected from the human antibody phage display library constructed in this study by using the respective proteins as antigens in the bio-panning. The success implies that the human antibody (2.6×10^8) repertoire was satisfactorily large. HuScFv proteins were induced to express by the *huscFv*-phagemid-transformed HB2151 *E. coli* clones. The whole cell lysates of these E. coli revealed multiple bands of HuScFv proteins as detected by using the mouse anti-E-Tag monoclonal antibody. The band multiplicity should be because some of the HuScFv were mature (without signal peptide and mostly located in the bacterial periplasm) while the others were still immature (with signal peptide and located in the cytoplasm). In addition to signal peptide, some of the HuScFv might have been degraded during the protein expression but the others were not depending upon the amino acid sequences of individual HuScFv and the rate of the recombinant protein expression of the particular *E. coli* clones even though they were grown under the same condition.

4. Binding of the HuScFv to the target venom components

The indirect ELISA, dot-ELISA, and Western blot analysis (WB) were used for testing the binding specificities of the HuScFv derived from phage bio-panning with different venom components.

In dot-ELISA, P1-P11 and the crude venom pool were individually dotted onto different NC membrane squares and they were allowed to react with individual HuScFv prepared from different transformed HB2151 *E. coli* clones. From the data of protein identification by 2D-LC/MS-MS, it was found that some protein peaks contained more than one component; thus, the HuScFv derived from bio-panning with one protein fraction might also react with components contained in other fractions as well and this was revealed by the results of the dot-ELISA. Another explanation for the results of dot-ELISA that HuScFv derived from bio-panning with one protein peak also bound to protein(s) in the other peak(s) might be due to the presence of the same peptide epitope in several proteins (shared epitope).

In WB, crude venom of *N. kaouthia* was separated on SDS-PAGE, trans-blotted onto NC membrane, and reacted with individual HuScFv preparations. Crude venom composed of several protein components with varying amounts, some are minute while the others are relatively abundant as revealed by proteomics in this study. This factor limited the presence of some epitopes of the minor proteins in the SDS-PAGE separated crude venom. Thus, some HuScFv clones that were specific to the minor proteins could not reveal the antigen-antibody reactive band(s) by the Western blot analysis. Moreover, the orientation of the same epitopes on NC membrane of the dot-ELISA (venom preparations were directly dotted onto the membrane) and the WB (SDS-treated) were different. With the awareness that the results of the two assays could be different, thus, the HuScFv from *E. coli* clones that could react with antigen either by dot-ELISA and/or by WB, were further used in the mimotopes searching.

5. *Mva*I RFLP analysis of *huscFv* of the page clones derived from bio-panning with P8 and the HuScFv mimotope identification

There were seven P8-specific HB2151 *E. coli* clones that expressed the HuScFv that could bind with the protein in the P8 which contained principally the long α-neurotoxin (designated clones P8/0/1, P8/9/1, P8/19/1, P8/7/2, P8/10/2, P8/22/3, and P8/31/3). The RFLP of the *huscFv* of these clones were studied and it was found that they had high diversity.

In mimotopes searching, phage clones display 12-mer peptides that were bound by the P8 derived HuScFv were recovered from the third round of bio-panning. The phage nucleotides were sequenced. Not all of the DNA sequencing were successful which implies that some of the phage genomic DNA did not contain the DNA insert that encodes the 12-mer peptide. One explanation is a competition among the phage particles to infect and grew in the *E. coli* host cells. Phages that lack DNA insert could grow faster than the phages that carried the inserted DNA sequences because all pIII of the former were available for binding to the F pili of the *E. coli* compared to the pIII of the latter which some had been used to display the 12-mer peptide partner. Moreover, the over expression of the peptide or peptide-fused- pIII is toxic to the *E. coli* host cells as has been demonstrated by Krebber *et al*. (Krebber *et al*., 1996).

Nevertheless, the amino acid sequences were deduced from the nucleotides of the clones that could be sequenced. All pf the clones, *i.e.,* clones P8/0/1, P8/9/1, P8/19/1, P8/7/2, P8/10/2, P8/22/3, and P8/31/3 revealed common peptide epitope: "TVNT" was homolog to TVKT peptide located at the loop-III of the *N. kaouthia* long α-neurotoxin. Asparagine (N) is an amino acid in the same group of lysine (K) that has hydrophilic side chain. Thus, it could be concluded that the epitope of the HuScFv to P8 that were produced is located in the loop-III of the *N. kaouthia* long α-neurotoxin which is the acetylcholine receptor binding domain of the *N. kaouthia* venom. The finding that the mimotopes of all HUScFv of different phage clones with different RFLP patterns were identical may simply reflects the affinity differences of the antibody fragments instead of the epitope binding differences.

6. Venom neutralization test

Long α-neurotoxin polypeptide of *N. kaouthia* folds into three-loops, Loops I-III, which are held together by five disulfide bonds and three β-sheets. Loops I and II are hydrophobic, especially the loop II which plays role in acetylcholine receptor (AChR) binding.

The TVNT mimotope of the HuScFv derived from bio-panning with the P8 was homologous to TVKT peptide located at the loop-III of long α-neurotoxin at position 47-50 which is involved in toxicity of long α-neurotoxin as discussed by Walkinshaw (1980). The chemical modification and toxicity study of invariant amino acids at Lys-49 in small loop III (amino acid 44-55 in **Figures 7** and **8**) are likely to be involved in AChR binding (for more details, please see Low, 1979 and Karlsson, 1979).

Moreover, not only loop III that has been shown to involve in the receptor binding of the long α-neurotoxin, loops I and II are also involved in the AChR binding. Mordvintsev *et al*. (2005) revealed by using computational molecular docking and molecular dynamic model that the nicotinic AChR (nAChR) interacted with the three short chain α-neurotoxins. The tip of loop-II inserts into the ligand-binding pocket between the α/γ or α/δ nAChR, while the loops I and III contact with nAChR in surface touch manner (**Figure 11**).

There have been several studies which indicated the role of the loop II in the receptor binding. Fruchart-Gaillard *et al*. (2002) identified the critical amino acid residues that bind with muscular type-acetylcholine receptor, *i.e.*, α7 AChR at high affinity. This type of receptor is consisted of homoheptamer. The critical amino acid residues located in the large loop II were: D27, F29, R33, K35, C26, C30, and R36 and at C-terminal tail, the F65 (**Figure 9**). Bourne *et al*. (2005) studied crystal structure of α-cobratoxin interacted with pentameric acetylcholine binding protein (AChBP) and showed that loop-II of α-cobratoxin was inserted into the interface of two subunits of AChBP (**Figure 10**).

In the *in vivo* venom neutralization test, the percentages of survived mice in the groups that received either single or multiple doses of the HuScFv specific to the long α-neurotoxin were equal to or better than that of the groups receiving the conventional horse anti-venom. The size of the HuScFv was ~27 kDa which is four times larger than the molecular size of the long α-neurotoxin (7 kDa). The therapeutic effect of the HuScFv may be either by the mere binding to the TVKT peptide of the loop-III or the binding of the HuScFv to the TVKT peptide might as well exert a steric hindrance of the loops I and II to the receptor. Either or both of these mechanisms should bring about the inaccessibility of the venom toxin to its target receptor.

The finding that the HuScFv specific to the acetylcholine receptor binding loop of the long α-neurotoxin alone were good therapeutic in the envenomized mice compared to the horse anti-venom which contained intact antibodies with multiple epitope specificities was encouraging. It is envisaged that the HuScFv cocktail with multiple venom component specificities should be a better alternative of the horse derived antibody preparation in terms of therapeutic efficacy, safely, and availability.