CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Animals

The animals used in the experiments (New Zealand white rabbits and mice) were supplied by Chenur Supplier (Selangor, Malaysia). The animals were housed in Laboratory Centre, Faculty of Medicine, University of Malaya, and received water and food *ad libitum* until use. The animals were handled according to CIOMS guidelines on animal experimentation (Howard-Jones, 1985).

3.1.2 Animal ethical clearance

The experimental protocol on animal study 2013-06-07/MOL/R/FSY was approved by the Animal Ethics Care and Use Committee, Faculty of Medicine, University of Malaya. (Animal ethical clearance is attached in Appendix K).

3.1.3 Venoms

Naja sumatrana venom was a pooled venom sample obtained from spitting cobras captured in central Malaysia and supplied by Snake Valley (Seremban, Malaysia).

Lyophilized *Naja sputatrix*, *Naja siamensis* and *Naja kaouthia* venom was purchased from Latoxan (Valence, France).

3.1.4 Antivenom

Neuro Polyvalent Antivenom (NPAV) (Batch No. NP00108, expiry date 21/4/2013) was supplied by Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand. The antivenom consisted of lyophilized powder of $F(ab')_2$ purified from hyperimmunized horses against venoms of four

Thai elapids: *Naja kaouthia*, *Bungarus candidus*, *Bungarus fasciatus*, and *Ophiophagus hannah*). Each vial of the antivenom lyophilized powder was reconstituted in 10 ml of steril normal saline before use, according to the instructions by the manufacturer.

3.1.5 Anesthesia

Anesthetic drugs, ketamine (dose: 35-50 mg/kg) and xylazine (dose: 5-10 mg/kg) were injected intramuscularly into rabbits.

3.1.6 Chemicals and general consumables

Opti-TOF ™ LC/MALDI INSERT (Applied Biosystem, California, USA) was on loan from University of Malaya Centre for Proteomics Research (UMCPR), Faculty of Medicine, University of Malaya.

ELISA Immunoplates (crystal grade polystyrene flat bottom, maxi-binding) were purchased from SPL Life Sciences, Korea.

Vivaspin® 15R Hydrosart was purchased from Sartorius Stedim Biotech GmbH, Goettingen, Germany.

Glass Econo Column for Low-pressure chromatography was purchased from Bio-Rad, Berkeley, California, USA.

Rabbit anti-horse IgG (H+L), $F(ab')_2$ fragments antibody, 2-(N-Morpholino) ethanesulfonic acid (MES) hydrate, Coomassie Brilliant Blue G-250, Coomassie Brilliant Blue R-250, phosphate buffered saline (PBS), phosphate buffered saline-Tween 20 (PBS-Tween), bovine serum albumin (BSA), acrylamide, N-N'-methylene bis-acrylamide, ammonium persulfate, glycerol, Freund's adjuvant, Freund's incomplete adjuvant, citric acid, horseradish peroxidase (100

purpurogalin unit/mg), sodium periodate, sodium acetate, sodium carbonate, sodium borohydride, o-phenylenediamine dihydrochloride, casein, trichloroacetic acid, calcium-bis-p-nitrophenyl phosphate, magnesium sulfate, sodium barbital, p-nitrophenylphosphate, 5'-adenosine monophosphate, trifluoroacetic acid (HPLC grade), ammonium molibdate, ascorbic acid, hyaluronic acid, calcium chloride, L-leucine, triethanolamine, o-dianisidine, acetylcholine iodide, dithionitrobenzoate, ammonium bicarbonate, α-cyano-4 hydroxycinnamic acid, Sephadex® G-25, Sephadex® G-50 gel beads and Orange G were purchased from Sigma-Aldrich, St. Louis, Missouri, USA.

2-D Clean-up kit, 2-D Quant kit, PlusOne DryStrip Cover fluid, HiTrap Protein A HP column (5 ml), Resource® S Ion exchange column (1 ml), dithiothreitol (DTT), iodoacetamide, urea, thiourea, CHAPS, IPG buffer (pH 3-10, Linear), Immobiline™ DryStrip (pH 3-10, 13 cm, Linear) were purchased from GE Healthcare Life Sciences, Little Chalfont, United Kingdom.

Sodium chloride, Tris, bromophenol blue, sodium dodecyl sulfate (SDS), glycine, deoxychloric acid sodium salt were purchased from MP Biomedicals, Santa Ana, California, USA.

Ethanol (analytical grade), phosphoric acid (analytical grade), hydrochloric acid, methanol (analytical grade), acetic acid (analytical grade) and formic acid (analytical grade) were purchased from Friendemann Schmidt, Washington, USA.

NN,N,N'-N'-tetramethylethylenediamine (TEMED), acetonitrile (HPLC grade), sodium dihydrogen monophosphate monohydrate, sodium hydroxide, sodium bicarbonate, ZipTip® Pipette tips, LiChrosphere® WP 300 C_{18} reverse-phase column cartridge were purchased from Merck, New Jersey, USA.

Hydrogen peroxide and sulfuric acid were purchased from J.T. Baker, Pennsylvania, USA.

Fermentas Spectra™ Multicolor Broad Range Protein Ladder and Pierce® Silver Stain for Mass spectrometry were purchased from Thermo Scientific, Waltham, Massachusetts, USA.

SDS-PAGE, Broad Range Molecular Weight Standards was purchased from Bio-Rad, Berkeley, California, USA.

Trypsin Gold, Mass Spectrometry Grade was purchased from Promega, Fitchburg, Wisconsin, USA.

iBlot® Gel transfer stacks, PVDF, mini, Novex® HRP chromogenic substrate (TMB) and UltraPure™ Agarose were purchased from Life Technologies, Carlsbad, California, USA.

Tween 20 was purchased from Biotium, San Francisco, USA. Ketamine (100 mg/ml) and xylazine (100 mg/ml) were purchased from Troy Laboratories Pty. Ltd, New South Wales, Australia.

Secondary antibody Goat anti-rabbit IgG-Horseradish Peroxidase (HRP) conjugates and rabbit anti-horse IgG $F(ab')_2$-Horseradish Peroxidase (HRP) conjugates were purchased from Abnova, Taipei, Taiwan.

3.1.7 Buffers

Tris-buffered saline (TBS) -Tween (1X)

Tris- buffered saline (TBS)-Tween (1X) was prepared from 50 mM Tris, 150 mM NaCl, and 0.05% (v/v) Tween 20 in UltraPure^{TM} water. The pH of the buffer was then adjusted to pH 7.6.

Citrate-phosphate buffer

Citrate-phosphate buffer (0.1 M), pH 5 was prepared by mixing 97.2 ml of 0.1 M citric acid and 102.8 ml of 0.2 M sodium phosphate.

3.2 GENERAL METHODS

3.2.1 Determination of protein concentration

3.2.1.1 Determination of protein concentration by Bradford method

Protein concentration of the crude venom or toxins was determined as described by Bradford (1976).

Bradford reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 ml of 95% (v/v) ethanol (analytical grade) and 100 ml of 85% (v/v) phosphoric acid then was added. The resulting solution was diluted to a final volume of 1 L. The final concentrations in the Bradford reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v) ethanol and 8.5% (v/v) phosphoric acid. The samples (crude venom or toxins) were reconstituted in appropriate buffer in a volume up to 0.1 ml and pipetted into 12 x 100 mm test tubes. A volume of 5 ml of Bradford reagent was added into each test tube and mixed thoroughly by inversion. The absorbance at 595 nm was measured after 2 min and before 1 h against a blank prepared from 0.1 ml of appropriate buffer and 5 ml of Bradford reagent. Bovine serum albumin (BSA) was used to construct the standard curve. The assay was carried out in triplicates.

3.2.1.2 Determination of protein concentration by 2-D Quant Kit

The determination of protein concentration for electrophoresis was done using 2-D Quant Kit as described by manufacturer. 2-D Quant Kit contains precipitant (250 ml), co-precipitant (250 ml), copper solution (50 ml), color reagent A (250 ml), color reagent B (5 ml) and a standard, bovine serum albumin (BSA, 2 mg/ml).

Prior to performing the assay, an appropriate volume of working color reagent was prepared by mixing 100 parts of color reagent A with 1 part of color reagent B. Each 2-D Quant Kit assay requires 1 ml of working color reagent.

One to fifty microliters of the samples was used for the assay. Five hundred microliters precipitant was added into each tube, mixed thoroughly and incubated at room temperature for 2-3 min. This was followed by adding 500 µl co-precipitant to each tube and mixed before centrifuged the tubes at 10,000 xg for 5 min. The supernatant was discarded without dispersion of the precipitated protein. One hundred microliters of copper solution and 400 µl of UltraPure™ water were added into each tube to dissolve the precipitated protein. One milliliter of working color reagent was added into each tube and mixed thoroughly. The mixture was incubated at room temperature for 20 min. The absorbance at 480 nm was measured against a blank.

A standard curve was prepared using BSA (0.5-50 µg proteins). The amount of protein (mg) was plotted against the corresponding absorbance value and used to determine the protein concentration of samples. The assay was carried out in triplicates.

3.2.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out under reducing condition according to the protocol described by Laemmli (1970). SDS-PAGE reagents were prepared as follows:

Solution A: 30% (w/v) acrylamide, 0.8% (w/v) N-N'- methylene bis-acrylamide

Solution B: 1.5 M Tris-HCl, pH 8.8

Solution C: 10% (w/v) sodium dodecyl sulfate (SDS)

Solution D: 10% (w/v) ammonium persulfate (APS)

Solution E: 0.5 M Tris-HCl, pH 6.8

SDS-PAGE electrophoresis buffer: 0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3

Sample loading buffer: 62 mM Tris-HCl, pH 6.8; 2.3% (w/v) SDS; 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.005% (w/v) bromophenol blue.

15% resolving gel was prepared by mixing 5 ml of solution A, 2.5 ml of solution B, 2.3 ml of UltraPure^{TM} water, 0.1 ml of solution C and 0.1 ml of solution D (0.1% (w/v) APS) and 10 µl of TEMED (N,N,N'N'- tetramethylethylenediamine) was added prior to casting the gel.

4% stacking gel was prepared by mixing 5 ml of solution A, 2.5 ml of solution E, 5.97 ml of UltraPure^{TM} water, 0.1 ml of solution C and 0.1 ml of solution D (0.1% (w/v) APS) and 10 µl of TEMED (N,N,N'N'- tetramethylethylenediamine) was added prior to casting the gel.

SDS-PAGE was carried out using Bio-Rad Mini Protean® Tetra system. The glass plates (80 mm x 100 mm) were assembled on gel caster according to instructions by manufacturer (Bio-Rad). Five milliliters of 15% resolving gel was loaded into the space. One milliliter of UltraPure™ water was added to overlay the resolving gel. The resolving gel was allowed to polymerize for 20 min at room temperature. After polymerization was completed, the overlaid UltraPure™ was discarded and 1 ml of 4% stacking gel was loaded. Bio-Rad 10 wells comb (1 mm) was immediately inserted into stacking gel and allowed to polymerize at room temperature. After polymerization, the glass plates were assembled into an electrophoresis tank, and filled with SDS-PAGE electrophoresis buffer. Electrophoresis was carried out at a constant voltage of 90 V for 1-2 h. Typically, 10-20 µg of protein was loaded into each well.

3.2.2.1 Preparation of protein samples

The protein samples were mixed with an equal volume of SDS loading buffer and heated in a boiling water bath for 5 min, prior to electrophoresis. Molecular weight markers (Fermentas, Spectra™ Multicolor Broad Range Protein Ladder and Bio-Rad, Broad Range, SDS-PAGE molecular weight standards) were used for calibration.

3.2.2.2 Fixing, staining and destaining

Upon completion of electrophoresis run, the plates were opened, and the gel was gently removed, and washed briefly in UltraPure™ water for 5 min before Coomassie Blue staining or silver staining.

Coomassie Blue staining

Coomassie Blue staining reagents were prepared as the following:

Fixing solution: 40% (v/v) methanol, 10% (v/v) acetic acid in UltraPure™ water.

Staining solution: 0.2% (w/v) Coomassie Blue R-250 in fixing solution.

Destaining solution: 5% (v/v) methanol, 7% (v/v) acetic acid in UltraPure™ water.

The gel was immersed in fixing solution for 5 min followed by staining with staining solution for 20 min. The gel was then destained in destaining solution until a clear gel was obtained.

Silver staining

Silver staining of the gel was carried out using Pierce® Silver Stain for Mass Spectrometry, according to manufacturer's protocol. The silver stain kit consists of Silver Stain Sensitizer (2 ml), Silver Stain Enhancer (25 ml), Silver Stain Developer (500 ml), and Silver Stain (500 ml).

Staining solutions were freshly prepared prior to use. Sensitizer working solution was prepared by mixing 1 part of Silver Stain Sensitizer with 500 parts of UltraPure™ water.

Silver mixture was prepared by mixing 1 part of Silver Stain Enhancer with 100 parts of Silver Stain. Developer working solution was prepared by mixing 1 part of Silver Stain Enhancer with 100 parts of Silver Stain Developer.

Others reagents were prepared as follows:

Fixing solution: 30% (v/v) ethanol, 10% (v/v) acetic acid

Ethanol rinse: 10% (v/v) ethanol

Stop solution: 5% (v/v) acetic acid

Briefly, the gel was washed twice in UltraPure™ water for 5 min. The UltraPure™ water was then discarded and fixing solution was added and left overnight. The gel was rinsed with ethanol twice for 5 min each. This was followed by rinsing in UltraPure™ water twice for 5 min each. The gel was incubated for 1 min with sensitizer working solution and subsequently rinsed with two changes of UltraPure™ water for 1 min each. Silver mixture was then added to the gel and incubated for 5 min. The gel was rinsed twice with UltraPure™ water for 20 sec each. Developer working solution was then added to the gel and incubated until protein bands appeared. When the desired band intensity was reached, developer working solution was discarded and replaced with Stop solution for 10 min. The gel was then kept in UltraPure™ water in 4°C until further use.

3.2.3 Production of antibodies to cobra venom and venom toxins

3.2.3.1 Immunization Schedule

Pre-immune serum was collected from the rabbits and used as control in ELISA. Antibodies to the venom and the venom toxins were prepared as follows:

In the first immunization, the venom samples (10 μg) or purified toxins samples (5 μg) were dissolved in PBS (pH 7.2) and mixed with an equal volume of Freund's complete adjuvant, before injected intramuscularly to the thigh of the rabbits (n = 3). For the subsequent immunizations, 20 μg of the venom samples and/or 10 μg of purified toxins samples were mixed with an equal volume of Freund's incomplete adjuvant and injected intramuscularly at multiple sites of the rabbit fortnightly for 8 weeks. Blood sample was collected in between to examine the antibodies titer by Indirect ELISA. Nine days after the final immunization, the rabbits were anesthetized by ketamine (35-50 mg/kg) and xylazine (5-10 mg/kg) according to standard anesthesia protocols (Muir *et al*., 2012) and bled by cardiac puncture for blood collection.

3.2.3.2 Purification of antibody immunoglobulin IgG

The blood collected from rabbits was allowed to clot at room temperature and centrifuged at 3,500 xg for 20 min. The serum was aliquoted and stored at -20°C until further use.

Proteins from serum were isolated and desalted by Sephadex® G-25 gel filtration column chromatography (30 mm x 480 mm) that had been pre-equilibrated with 0.05% (v/v) acetic acid. The same acetic acid solution was used as eluent. The flow rate was 30 ml/h and fractions of 5 ml were collected. Protein content was measured by absorbance at 280 nm. The fractions were pooled and lyophilized with Heto FD4 Lyophilizer (Cambridge Scientific).

The IgG proteins were purified from desalted protein samples using HiTrap Protein A HP affinity prepacked column (GE Healthcare), using the ÄKTA purifier 10. The column was first equilibrated with binding buffer, 20 mM sodium phosphate, pH 7 for 10 column volumes, at a flow rate of 5 ml/min. The desalted protein samples were dissolved in binding buffer and loaded to the Protein A affinity column. The purification was carried out by washing with 5 column volumes of binding buffer into the column, followed by elution of IgG with 5 column volumes of elution of buffer (0.1 M citric acid, pH 3) at a flow rate of 5 ml/min. The pH of IgG fractions collected was adjusted by adding 100 µl of 1 M Tris-HCl, pH 9.0 per ml of fractions. The purified IgG fractions were kept at -20°C until further use.

3.2.3.3 Conjugation of horseradish peroxidase (HRP) to the purified IgG

Antibody IgG-HRP conjugate was prepared according to the method described by Wisdom (1996), with slight modifications.

Four milligrams of horseradish peroxidase (100 purpurogalin unit/mg) was dissolved in 1 ml of UltraPure^{TM} water, and 200 µl of freshly prepared 0.1 M sodium periodate was added. The solution was stirred for 20 min at room temperature. The resulting HRP-aldehyde was dialyzed against 1 mM sodium acetate buffer, pH 4.4, overnight at 4°C, with several changes of buffer. The pH of the dialyzed enzyme solution was adjusted to 9.0-9.5 by adding 20 µl of 0.2 M sodium carbonate buffer, pH 9.5 followed immediately by addition of 8 mg of IgG (dissolved in 1 ml of 0.01 M sodium carbonate buffer, pH 9.5) and stirred for 2 h at room temperature. The Schiff's base was reduced by adding 50 µl of freshly prepared sodium borohydride solution (4 mg/ml), The mixture was stirred for 2 h at 4°C. The conjugate was then dialyzed overnight against several

changes of PBS (pH 7.2) at 4°C. The IgG-HRP conjugate was aliquoted and stored frozen until required.

3.2.4 Indirect Enzyme-linked Immunosorbent Assay for measurement of antibodies titer

3.2.4.1 Chequerboard titrations

Preliminary chequerboard titrations to determine the optimum working dilutions of antibodies were carried out using goat anti-rabbit IgG-HRP conjugate at a dilution of 1:6000 in PBS-Tween (pH 7.2).

Titration curves were obtained using varying concentration of venom or toxins antigens from 5 to 100 ng/ml in 0.05 M sodium carbonate buffer (pH 9.5). The optimum working dilutions of antibodies were chosen when absorbance at 492 nm was 0.8-1.0.

3.2.4.2 Assay Procedure

ELISA immunoplate (SPL) was coated with 100 ng/ml of venom or venom toxin as antigen in 0.05 M sodium carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plate was washed 4 times with 200 µl PBS-Tween, followed by the addition of 100 µl of antiserum collected during immunization (dilution of 1:200). Similar dilution of pre-immune serum was used as control for Indirect ELISA assay. The plate with antiserum was incubated for 1 h at room temperature. After washing 4 times with PBS-Tween, 100 µl of goat anti-rabbit IgG-HRP conjugate at dilution of 1:6000 was added into the wells and incubated for 1 h at room temperature. The plate was washed 4 times with PBS-Tween before adding 100 µl of substrate (0.4 mg/ml of o-phenylenediamine dihydrochloride in 0.1 M citrate-phosphate buffer, pH 5 containing 0.003% (v/v) H_2O_2) for color development. The enzymatic reaction was allowed to proceed for 30 min at room temperature in dark and stopped by adding 50 µl of 12.5%

(v/v) H_2SO_4. The absorbance at 492 nm was determined using Bio-Rad Model 690 microplate reader.

3.2.5 Determination of the median lethal dose (LD_{50}) of venom and toxins

The intravenous median lethal dose (LD_{50} *i.v.*) of the venom was determined by injecting appropriate dilutions of the sample intravenously into the caudal vein of mice (n = 4, 18-20 g). The survival ratio was determined after 24 h. The LD_{50} (95% confidence interval, C.I.) was then calculated by the Probit analysis (Finney, 1952).

The intramuscular median lethal dose (LD_{50} *i.m.*) of the venom was determined by injecting various amount of the venom into the thigh muscle of mice (n = 4, 18-20 g). The survival ratio was determined after 24 h. The LD_{50} (and its 95% confidence interval, C.I.) was then calculated by Probit analysis (Finney, 1952).