CHAPTER 2

EXPERIMENTAL

2.1 Material

2.1.1 Snake Venom

Snake venom of *Trimeresurus macrops* was obtained from the Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand. The crude venom was lyophilized and stored at 4°C before use.

2.1.2 Chemicals and Apparatus

Most electrophoresis chemicals used in this research were the highest quality grade available and from Amersham Pharmacia Biotech (Uppsala, Sweden). For gel filtration media, Sephacryl S-100 High Resolution media were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). HPLC grade methanol and acetonitrile (LabScan, Dublin, Ireland) were used. Water used in this research was double distilled (GFL Glass Water Sills, Germany) and filtered through a 0.45 μm filter. For mass spectrometric analysis, matrix and standard peptides were purchased from Sigma (Sigma Chemicals, USA). For necessary apparatus, there are protein macrotrap desalting cartridge (Michrom Bioresource), laboratory centrifuge (Biofuge pico Heraeus; Kendro, Germany), micropipettes adjustable from 2 to 1,000 μl (Gilson, France), pipette tips (Bioline, USA), eppendroff (Axygen Scientific, USA), vortex mixer (Vortex-Genie2, Scientific Industries, USA), orbital shaker (Kika-Werke GMBH&Co., germany), sonicator (BHA-1000, Branson, USA), freeze dryer (Labconco, USA), speed vacuum centrifuge (Heto-Holten, Denmark).

2.2 Purification Methods

2.2.1 SDS-Polyacrylamide Gel Electrophoresis of Snake Venom

1) SDS-PAGE

SDS electrophoresis was performed according to Laemmli (1970).[33] SDS-polyacrylamide gels were cased and run in the compact Hoefer miniVE Vertical Electrophoresis system (Amersham Pharmacia Biotech). The SDS-PAGE procedure was performed exactly as described in Method 8 (Vertical PAGE) of Electrophoresis in Practice text book. The stock solutions are 40%*T*, 3%*C* acrylamide, bis solution (40% acrylamide, 1.2% *N,N'*-methylenebisacrylamide), stacking gel buffer(0.5 M Tris-HCl, pH 6.8, 0.4% SDS), resolving gel buffer (1.5 M Tris-HCl, pH 8.8, 0.4% SDS), 40% ammonium persulfate solution (APS), cathode buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS). The SDS-polyacrylamide gels (8 x 9 cm gels; 1mm thick; 10 wells) were prepared as discontinuous gel. For two discontinuous gels, the composition of 12%*T*, 3%*C* resolving gel solutions are 2.0 ml of glycerol, 2.4 ml of 40%*T*, 3%*C* acrylamide, bis solution, 2.0 ml of resolving gel buffer, 1.6 ml of water, 8 µl of 40% APS, and 4 µl of TEMED. The 5%*T*, 3%*C* stacking gel solutions are 0.5 ml of acrylamide, bis stock solution, 1.0 ml of stacking gel buffer, 2.5 ml water, 4 µl of 40% APS, and 2 µl of TEMED. First, 3.4 ml of resolving gel solution was pipetted into the gel cassette. Then, 1.2 ml of stacking gel solution was carefully applied like an overlay. The comb was inserted without trapping air bubbles for making the sample wells. These two gel solutions were polymerized together for one day before use. For sample preparation, the 0.3 mg of *T. macrops* venoms was dissolved in 300 µl of Tris buffer (10mM Tris-HCl, pH 7.3, 50 mM NaCl). The resulting solution was centrifuged at 5000 rpm for 15 min; the supernatant was collected and used as the venom stock solution. The 26 M reducing sample buffer contains 50 µl of 2.6 M dithiothreitol (DTT; 30 mg of DTT in 60 µl water) and 5 ml of non-reducing sample buffer (1.0 g of SDS, 3 mg of EDTA, 10 mg of bromophenol blue, 2.5 ml of resolving gel buffer, 25 ml glycerol, make up to 100 ml with water). The diluting samples were prepared from the mixture of 5 µl of the venom stock solution and 23.75 µl of the 26 M reducing sample buffer for loading gel samples and the contents of a vial of Low Molecular Weight (LMW; molecular mass range 14.4 to 97.0 kDa)

SDS Calibration kit (Amersham Pharmacia Biotech) was dissolved in 415 µl of the 26 M reducing sample buffer for LMW marker. Then, the diluting samples were boiled for 3 min. After cooling, 1 µl of 2.6 M DTT was added to each sample. Before electrophoresis starting, the comb was removed from gel, the cathode buffer was poured in to buffer chamber, 10 µl of diluting samples were loaded into the wells using the gel loading tip (Bio-Rad, USA), the safety lid was placed, and the system was connected to power supply. The electrophoresis was carried out at 280 V, 20 mA, and 6 W for one gel and then was terminated when the dye front reached close to the bottom of the gel.

2) Coomassie Staining

The colloidal staining was used because this method has high sensitivity.[12] The staining procedure consists of the five steps and all steps using the orbital shaker. After finishing the electrophoresis, the gel was removed and then placed in the staining plastic box. The first step was the gel fixing in 12% (w/v) TCA for 1 hour. The staining step was the second step took overnight with 160 ml of staining solution (0.1% w/v Coomassie G-250 (Sigma, USA) in 2% H_3PO_4, 10% w/v ammonium sulfate) plus 40 ml of methanol (add during staining). The third step was the gel washing in 0.1 M Tris-H_3PO_4 buffer at pH 6.5 for 3 min. Then, the gel was rinsed in 20% v/v aqueous method for 1 min. Finally, the stabilizing step was the preserving gel in 20% aqueous ammonium sulfate.

2.2.2 Two-Dimensional Electrophoresis of Snake Venom

1) IEF and SDS-PAGE

The 2-D electrophoresis separation was performed as described in the manual of 2-D Electrophoresis using immobilized pH gradients.[15] For the first-dimension IEF, the venom stock solution was mixed with the lysis buffer (8 M urea, 4% CHAPS, 2% IPG buffer pH 3-10 or 4-7). Then, this sample solution was mixed with the rehydration solution (8 M urea, 2% CHAPS, 2% IPG buffer pH 3-10 or 4-7, 0.2% DTT, 0.002% bromophenol blue). The rehydration solution with a sample solution was applied on pH 3-10 and 4-7 IPG strips (length 7 cm). The IPG strip was

positioned on the Immobiline DryStrip Reswelling Tray and allowed to rehydrate overnight at room temperature. The rehydrated IPG strip was removed from the reswelling tray and transferred to the Immobiline Drystrip aligner. The IEF electrophoresis was carried out at 20°C on the Multiphor II Electrophoresis Unit with Immobiline Drystrip Kit (Amersham Pharmacia Biotech). The running condition of IEF was as follows: 200 V for 1 min, 3500 V for 1.5 h, then 3500 V for up to 3.7 kVh (for pH 3-10 IPG strip) or 3500 V for up to 5 kVh (for pH 4-7 IPG strip). After IEF, the IPG strip was equilibrated for 15 min in the equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT, 0.002% bromophenol blue), then equilibrated 15 min in the equilibration solution that replaced DTT with 2.5% iodoacetamide. Each IPG gel strip was embedded on the top of the SDS-polyacrylamide gel and covered with 0.5% agarose. For the second-dimension electrophoresis, SDS-polyacrylamide gel was run in the Hoefer miniVE Vertical Electrophoresis system. The SDS-PAGE was carried out on a 12.5%*T*, 2.6%*C* SDS-polyacrylamide gel (8 x 9 cm, 1 mm thick) at 280 V, an initial current 10 mA/gel for 15 min, then 20 mA/gel until the bromophenol blue dye front reached the bottom of gel. The 12.5%*T*, 2.6%*C* SDS-polyacrylamide gel was prepared from the polymerization of the gel solution (4.17 ml of 30%*T*, 3.6%*C* acrylamide, bis solution, 2.5 ml of 1.5 M Tris-HCl, pH 8.8, 0.1 ml of 10% SDS, 3.18 ml of water, 50 µl of 10% APS, and 3.3 µl of TEMED). The LMW Calibration Kit was used as standard molecular weights marker proteins.

2) Protein Detection

For 2-D Analytical gels, The Coomassie staining and Silver staining methods were used.[12, 15] The colloidal Coomassie staining procedure was described above in Topic 2 of Section 2.2.1. For Silver staining method, analytical gels were fixed with 40% ethanol and 10% acetic acid solution for 1 h. The gels were sensitized with 30% ethanol, 5% sodium thiosulfate, 6.8% sodium acetate, and 25% glutardialdehyde. The gel was washed with distilled water for 5 min and this wash step was repeated 3 times. Then, the gel was stained with freshly made silver solution (2.5% silver nitrate and 37% formaldehyde for 1 h and washed with distilled water. The gel was developed in 2.5% sodium carbonate and 37% formaldehyde) for 5 min. Staining was halted with 1.5% EDTA-Na_2 solution for 10 min. The gel was washed

with distilled water for 5 min then the step was repeated for 3 times. Finally, the gel was preserved in 87% glycerol for further analysis.

3) In-gel Digestion

The protein spots were manually excised from the SDS-polyacrylamide gels and transferred to 0.6 ml eppendroffs. This In-gel digestion protocol had been modified from the method described in Chapter 6: the preparation of protein digests for mass spectrometric sequencing of the book of Protein Sequencing and Identification Using Tandem Mass Spectrometry.[18] The gel pieces were washed twice with 20 µl of water for 15 min each. Then, the gel pieces were washed twice with 100 µl of 50%ACN/0.1 M ammonium bicarbonate for 20 min each at 30°C and dried using a speed vacuum centrifuge. Dried gel pieces were swollen in 100 µl of 10 mM DTT /0.1 M ammonium bicarbonate /1 mM EDTA and incubated at 60°C for 45 min and then the excess DTT solution was removed. The 100 µl of 100 mM iodoacetamide /0.1 M ammonium bicarbonate was added and allowed to stand in the dark at room temperature for 30min. The iodoacetamide solution was removed. The gel pieces were washed twice with 100 µl of 50% ACN/0.05 M Tris-HCl, pH 8.5 for 5 min each and dried. Dried gel pieces were swollen in 100 µl of trypsin (Sigma) solution (10 µl of trypsin in 1% acetic acid and 90 µl of trypsin buffer containing 50 µl of 0.1 M Tris-HCl, pH 8.5, 1 µl of 100 mM $CaCl_2$, 10 µl of ACN, and 39 µl of distilled water) and incubated at 4°c for 45 min and then the excess solution was removed. The gels were added with 10 µl of trypsin buffer and incubated overnight at 37°C. The trypsin reaction was stopped with adding 10 µl of 2% TFA at 60°C for 30 min. The supernatant was collected and transferred to 0.6-ml eppendroff. The peptides were subsequently extracted three times with 20 µl of 0.05 M Tris-HCl/1 mM $CaCl_2$, 20 µl of 50% ACN/25 mM Tris-HCl/0.5 mM $CaCl_2$, and 20 µl of 2.5% formic acid/50% ACN. The extraction steps were incubated at 30°C for 10 min, sonicated for 2-3 min, and then collected supernatant from each step. All supernatants were combined and reduced the volume of solution to 20 µl using a speed vacuum centrifuge. These solutions were kept at -20°C for further analysis.

2.2.3 Gel Filtration Chromatography of Snake Venom

T. macrops venom (5.5 mg) was dissolved in 0.7 ml of 20 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl. The solution was centrifuged at 12,000 rpm for 3 min and then the supernatant was collected. Gel filtration was performed using AKTAprime system comprising pump, UV detector, and fraction collector (Amersham Pharmacia Biotech). 0.5 ml of supernatant was injected to a column of Sephacryl S-100 HR column (1.6 x 52.5 cm) equilibrated with the Tris-HCl buffer. The column was eluted at flow rate 0.5 ml/min with the same buffer and maintained at 4°C. The protein absorption was monitored at 280 nm. The fractions were collected and concentrated using a freeze dryer, and then kept at -20°C.

2.3 Assays

The gel filtration fractionated proteins were used for enzymatic activity determinations. The purified proteins were resuspended in 100 µl of 50 mM Tris-HCl buffer (pH 8.0). The protein concentration of venom fractions was determined by measuring the absorbance at 280 nm, based upon the assumption that the absorbance of 1mg/ml of crude venom was 1.600.

2.3.1 Proteolytic Activity

Proteolytic activity was determined using heat-denatured casein as substrate.[34] 0.5 ml of 2% casein (Merck, USA) in 50 mM Tris buffer at pH 8.0 and 25 µl of a sample solution was incubate at 37°C for 30 min. The undigested casein was precipitated and reaction terminated by adding 0.5 ml of 5% TCA. After centrifugation at 10,000 rpm for 10 min, the absorbance of the supernatant was measured at 280 nm. One unit of activity was defined as the amount of enzyme hydrolyzing casein at such an initial rate that the amount of TCA soluble products give an absorbancy of 1.0 at 280 nm per 30 min.

2.3.2 Phospholipase A Activity

Phospholipase A activity was determined by the indirect hemolytic method in agarose plate according to Gutierrez et al. (1988).[35] Human erythrocyte suspension was used in place of the sheep erythrocytes in the original method.[31, 36] For preparation of agarose plate, 25 ml of 1% agarose in phosphate buffer saline (pH 7.2) containing 0.02% NaN_3, 0.5 ml of Red Blood Cell suspension (0.1ml of NaN_3-citrate and 1 ml of human blood), 1.0 ml of egg yolk in phosphate buffer saline (pH 7.2), and 0.25 ml of 10 mM $CaCl_2$ were mixed at 50°C and poured in a plastic disc (diameter 13.5 cm). The agarose plate was pierced for making of 21 sample wells per plate. 5 µl of sample solution and 1.0, 0.3, 0.1, 0.03 and 0.01 mg/ml crude venom were loaded. After 24 h, the diameters (mm) of clear zone were measured.

2.4 Protein Identification Methods

2.4.1 MALDI-MS

Mass spectra of protein and peptide were acquired using a MALDI/Tof mass spectrometer (BIFLEX, Bruker, Germany) operating in linear and reflectron modes. The dried samples were dissolved in 50% ACN/ 0.1% TFA and vortexed. The dried droplet method was used for MALDI-MS sample preparation.[19] α-Cyano-4-hydroxycinnamic acid (CCA) was used as the matrix. For proteins, 1 µl of a sample solution was mixed with 1 µl of a matrix solution (saturated CCA in 50% ACN/ 0.1% TFA). For peptide mixtures, 1 µl of sample solution was mixed with 5 µl of a matrix solution (saturated CCA in 50% ACN/ 0.1% TFA). Then, 1 µl of the mixture solution was spotted on MALDI target and allowed to dry at room temperature. The MALDI protein mass spectra were performed using a linear mode and calibrated using myoglobin (average mass 16951 Da) and bovine serum albumin (average mass 66433 Da). MALDI peptide spectra were performed in reflectron mode and internally calibrated using angiotensin II (average mass 1047.2 Da) and insulin B chain oxidized form (average mass 3495.9 Da). Peptide mass mapping obtained from each digested protein were searched against protein database via the MASCOT program (www.matrixscience.com). Search parameters allowed for carbamidomethylation of

cysteine, two missed trypsin cleavages, and 100 ppm mass accuracy. Peptides in the mass range of 800-3500 Da were selected for database search.

2.4.2 N-Terminal Sequence Determination

The purified proteins from a gel filtration method were analyzed. Desalting of gel filtration fractionated proteins were performed using reusable reversed-phase cartridge (Protein Trap, Michrom Bioresource). The desalted protein was dried using a freeze dryer. The dried sample was dissolved in 50%ACN/0.1% TFA. The N-terminal sequence determination was performed by Edman degradation using a sequencer (Applied Biosystems Model 473A, Germany). The obtained amino acid sequence was searched using BLAST program (www.ncbi.nlm.nih.gov/blast/).

2.4.3 Tandem MS

Tandem mass spectrometric analysis was performed on the ESI-Q-Tof mass spectrometer (Micromass, Manchester, UK). The desalted GF fractionated protein was tryptic digested by in-solution digestion without reduction and alkylation. The tryptic peptides were analyzed. The tandem MS spectra were searched against a protein sequence database using the MASCOT program (www.matrixscience.com). The search parameters were trypsin enzyme, 50 ppm mass accuracy, doublet and triplet charged peptides, ESI-Q/Tof instrument.