CHAPTER 3

EXPERIMENTAL

3.1 Snake venom

Crude *Bungarus candidus* venom (10 mg/mL in a phosphate buffer 200μL) and *Bungarus candidus* venom ion exchange fractions were obtained from the Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand. All of them were stored at -20 ^{o}C prior to use.

Bungarus candidus venom fractions 2, 3, 4, 6, 7 and 8 were fractionated from ion exchange chromatography. Crude venom (50 mg) was dissolved in 2 mL of 50 mM phosphate buffer at pH 6.25 then applied on resource^{TM} S column (Amersham Pharmacia biotech) 6 c.m. and eluted with 200 mL linear gradient 0-0.25 M NaCl phosphate buffer with flow rate 20 mL/hr.

3.2 Chemicals

Most reagents and staining kit used in SDS-polyacrylamide gel electrophoresis were purchased from Plusone Pharmacia Biotech (Uppsala, Sweden). The low molecular weight calibration kit was used as standard molecular weight marker proteins and purchased from Amersham Pharmacia biotech (UK). Brilliant Blue G and trichloroacetic acid for staining gels were obtained from Sigma (U.S.A.) and Merck (Germany), respectively.

Solvents used for in-gel digestion and preparation for mass spectrometric and sequencing techniques were HPLC grades and were purchased from Amersham Pharmacia biotech (Uppsala, Sweden), Labscan (Bangkok, Thailand) and Merck (Germany), respectively. Double distilled water was used in this research was prepared with Glass water Sills (GFL Gesellschaft für Labortechik mbH, Germany).

3.3 Apparatus and Instrument

Immobilized pH gradient strips (IPG strips) pH 4-7 (Amersham pharmacia biotech) were run in Multiphor™ II Electrophoresis Unit (Amersham pharmacia biotech, Uppsala Sweden). SDS-polyacrylamide gel electrophoresis gels were run in HoeferTM miniVE (minivertical), 8x9 cm gels (Amersham pharmacia biotech, Uppsala, Sweden).

Desalting Cartridge (Protein macrotrap, Michrom BioResource Inc.) was used for desalt *Bungarus candidus* venom fraction 6 and 8.

N-terminal sequencing of proteins in *Bungarus candidus* fractions 6 and 8 was done by Applied Biosystems 473 A Protein Sequencer.

Mass spectra of molecular weight of proteins were recorded on Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometric (MALDI-TOF MS) (BIFLEX Bruker, Germany). Mass spectra of sequencing protein were acquired from Electrospray Quadrupole-Time of flight (Model Q-TOF II, Micromass, UK). 1.5 and 0.5 siliconized eppendorf and pipette tips were purchased from Axygen sciencetific Inc. and Bioline U.S.A. ,respectively.

The other apparatus and instrument used in this research were:

- Micropipette

  pipetteman, Gilson, France.
- Vortex mixer

  Vortex-genie2, Sciencetific Industries.
- pH meter

  Denver Instrument U.S.A. system
- speed vacuum

  MAXI dry plus, Heto vacuum centrifuge, Denmark:
- sonicate

  DHA-1000; Branson, U.S.A.

3.4 Procedures

3.4.1 SDS-PAGE[26]

The glass gel cassettes were prepared, and then 20 %T SDS-PAGE was performed. Firstly, acylamide, bis acrylamide solution (40%T, 3%C) 6.67 mL was added into a beaker, then 1.5 M Tris-HCl pH 8.8 2.5 mL was added into the solution. After that, 10%SDS 0.1 mL was added to the solution and double distilled water 0.68 mL, and then mixed the solution. TEMED 3.3 µL and 50 µL of 10 % ammonium persulfate were added and the beaker was gently swirled to mix. The gel solution was poured into the gel cassette and waited for the polymerization. Lastly, 25 mM Tris, 192 mM glycine pH 8.8 containing 0.1 % SDS was used as an electrode buffer.

3.4.2 2D-Gel electrophoresis[26]

Crude venom (10 mg/mL) 15 µL (for coomassie staining gel, 25µL) was solubilized in 10 µL lysis buffer (8 M urea, 4% CHAPS, 2% IPG buffer pH 4-7) and pipette 200 µL rehydrate solution (8 M urea, 2 M thiourea, 4% CHAPS, 60mM DTT, 2% IPG buffer pH 4-7, 0.002% bromophenol blue) then applied on reswelling tray and put pH 4-7 IPG strips on the solution, then allowed the IPG strips to rehydrate at room temperature for 10 hours.

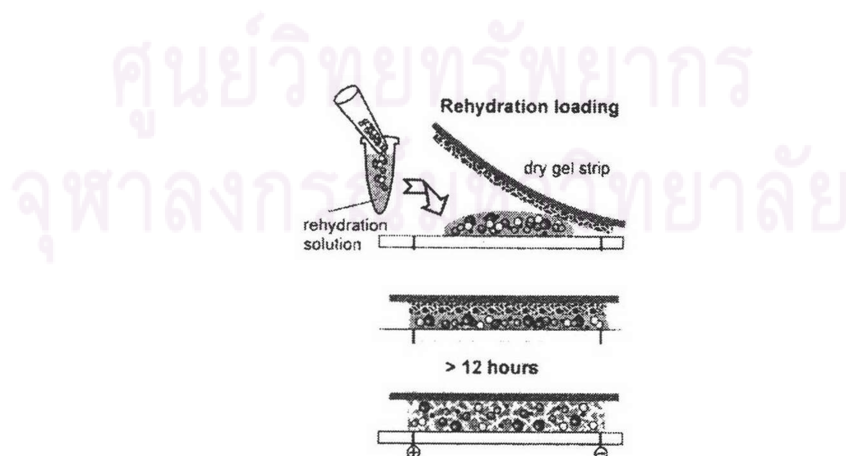

Figure 3.1 Scheme representations to apply the sample into IPG strips

After that, IPG strips were run on Multiphor™ II Electrophoresis machine. The running condition of isoelectric focusing (IEF) was as follow: 200V for 0.001kVh, 3500V for 2800 kVh and 3500V for 5200 kVh. After IEF, each IPG strip was equilibrated for 15 minutes in the equilibration solution (2 % SDS, 50mM Tris-HCl pH 8.8, 6M urea, 30 %(v/v) glycerol, 0.002 % bromophenol blue, 2% DTT), then equilibrated for 15 minutes in the equilibration solution that replaced DTT with 2.5% iodoacetamide. Each IPG strip was embedded on top of the 2-D gel and covered with 0.5% agarose. Second-dimensional SDS-PAGE was carried out on 20%T acrylamide gel. The running condition was as follow: 10 mA/gel for 15 minute and 20 mA /gel until the bromophenol blue dye front reached the bottom of the gel. Lastly, the each gel was stained by silver stained or coomassie brilliant blue G stained.

3.4.3 1-D gel electrophoresis

Each sample was added in a sample buffer (50 mM Tris-HCl pH 6.8, 0.1 M DTT, 10 % glycerol, 2 % SDS and 0.1 % bromophenol blue) After that, the solution was boil for 2 min., and added with DTT solution 1 µL. The solution was loaded into 20%T polyacrylamide gel wells. The SDS-PAGE gel was run in HoeferTM miniVE at 20 mA/gel until the bromophenol blue dye front reached the bottom of the gel. After completion of electrophoresis, the gels were stained with coomassie brilliant blue G. The low molecular weight calibration kit was used as standard molecular weights marker proteins.

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3.4.4 Staining method

1) Silver staining [26]

After electrophoresis, the gel was covered with 250 mL of 80% (w/v) ethanol, 1% aceteic acid glacial for 30 minutes. The solution was removed and covered the gel with 250 mL of 30 % (v/v) ethanol, 0.2 % (w/v) sodium thiosulfate, glutardialdehyde and 6.8 % (w/v) sodium acetate. Then, the gel was washed by distilled water for five minutes three times. After that, the gel was covered with 250 mL of 0.25% (w/v) silver nitrate and 0.0148 % (w/v)

formaldehyde. The gel was washed by distilled water for one minute two times. The solution of 250 mL of 2.5 % (w/v) sodium carbonate and 0.007 % (w/v) formaldehyde was added to the gel for 5 minutes. Next, the solution was removed and the gel was covered with 250 mL of 1.46 % (w/v) EDTA-Na_2•$2H_2O$. The gel was washed by distilled water for 5 minutes 2 times, and was stored in distilled water before excised the gel.

2) Coomassie staining[15]

After electrophoresis, the gel was covered with 250 mL of 20 % (w/v) trichloroacetic acid for 1 hour. The solution was remove and covered the gel with the colloidal coomassie blue staining solution and stain overnight at room temperature. Then, staining solution was removed and the gel was washed by 250 mL of 25% (v/v) methanol. After that, the gel was stored in 250 mL of the 20% (w/v) of ammonium sulfate.

3.4.5 In-gel digestion[15]

Protein bands or spots were manually excised from the polyacrylamide gels and transferred to 500 μL siliconized eppendorfs. The gel pieces were washed three times with 150 μL of 50% ACN/ 0.1M ammonium bicarbonate for 30 minutes. The gel pieces were dried using a speedvac concentrator. Dried gel were swollen in 0.1M NH_4CO_3/10mM DTT/ 1mM EDTA and incubated at 60 °C for 45 minute then remove the supernatant and dried gels. Gels were added 100mM iodoacetamide/ 0.1M NH_4CO_3 solution and incubated at room temperature in the dark for 30 minutes then remove the supernatant. After that gels were washed with 50% ACN three times and dried. Dried gels were swollen in 50 μL of 0.05M Tris-HCl pH 8.0/ 100mM $CaCl_2$ containing 5 μg trypsin (Promega). Gel pieces were incubated at 37 °C for over night and the reaction was stopped with formic acid 3 μL. Peptides were extracted twice with 50 μL of 0.05M Tris-HCl pH 8.0/ 10% ACN and 50 μL of 50% ACN. The extracted solutions were combined and concentrated by using a speedvac concentrator.

3.4.6 Sample preparation for MALDI-TOF

One microlittre sample was mixed with 10 μL matrix solution (5-10 mg of α–cyano-4-hydroxy-cinnamic acid in 50% ACN/ 0.1%TFA) in eppendorf 0.5 mL and spotted on the sample plate. (Myoglobin (1mg/mL) and angiotensin II (1mg/mL) were used for external calibration.)

3.4.7 Sample preparation for Edman degradation and MS/MS

The reusable reversed-phase cartridge was equilibrated with 0.1% formic acid in water 300 μL. *Bungarus candidus* venom fraction (6 or 8), 200 μL was loaded on the column slowly. Then, the salts were eluted with 400 μl of the acetonitrile: water (10:90) solution. The protein was eluted with 0.1 % (v/v) formic acid in 400 μL acetonitrile: water (90:10) solution and dried by speed vacuum centrifuge.

3.4.8 Protein Identification

Peptide mass spectra and amino acid sequence were acquired using MALDI-TOF and ESI Q-TOF mass spectrometer, respectively. Peptide mass fingerprints (PMF) spectra were obtained from each digested protein were searched database *via* PepIdent (http://au.expasy.org/tools/peptident.html) and MASCOT (http:// www.matrixscience.com). The peptide sequences were used for protein identification *via* Blast (http:// www.ncbi.nlm. nih. gov/ BLAST/Blast.cgi).