CHAPTER III

METERIALS AND METHODS

3.1 Instruments

Aggregometer

-Chronolog Lumi-Aggregometer™

Centrifuge

-Beckman Avanti™ 30, Beckman, USA

Electrophoretic Titration (ET)

-Pharmacia gel system, Pharmacia, Sweden

Filter

-Millex HV , Millipore, USA

-Acrodisc®, Pall cooperation, USA

-Centricon, Millipore, USA

Height Performance Liquid Chromatography (HPLC)

-Detector 2487 dual λ WATERS, USA

-Pump 1525 binary HPLC pump WATERS, USA

Hydrophobic interaction chromatography (HIC) column

- HIC PH-814 column, Shodex®, Japan

Lyophilizer

-LABCONCO, USA

Mass spectrometer

-AUTOFLEX II-TOF/TOF, Bruker Daltonics, German

Reverse phase column

-C18, Vydac™, USA

Size exclusion column

-Waters protein-pak™ 125 WATER S, USA

-Waters protein-pak™ 60 WATER S, USA

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

-Power supply, Pharmacia phast system™, Sweden

-12% SDS gel kit, invitrogen™, USA

-Stain kit, invitrogen™ SimplyBlue™, USA

X-ray film

- Kodak X-OMAT, Kodak, USA

3.2 Chemicals

Crude venom, Thai Red Cross society, Bangkok, Thailand

Human fibrinogen, Sigma, USA

Insulin b chain, Sigma, USA

Opossum serum, Kleberg Country Animal Control official, Texas, USA

Thrombin, Sigma, USA

Trypsin, Sigma, USA

3.3 Methods

3.3.1 Crude venom collection

Pooled Malayan pit viper (*Calloselasma rhodostoma*) venom was extracted from snakes which maintained at the Queeñ Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand. The snakes were bred and housed at the Thai Red Cross serpentarium. The snakes were allowed to bite into a nylon cloth membrane over a beaker and the venom was immediately transferred to eppendorff tube. The pooled venom was lyophilized. Crude Malayan pit viper from Vietnam was obtained from venom supplies.

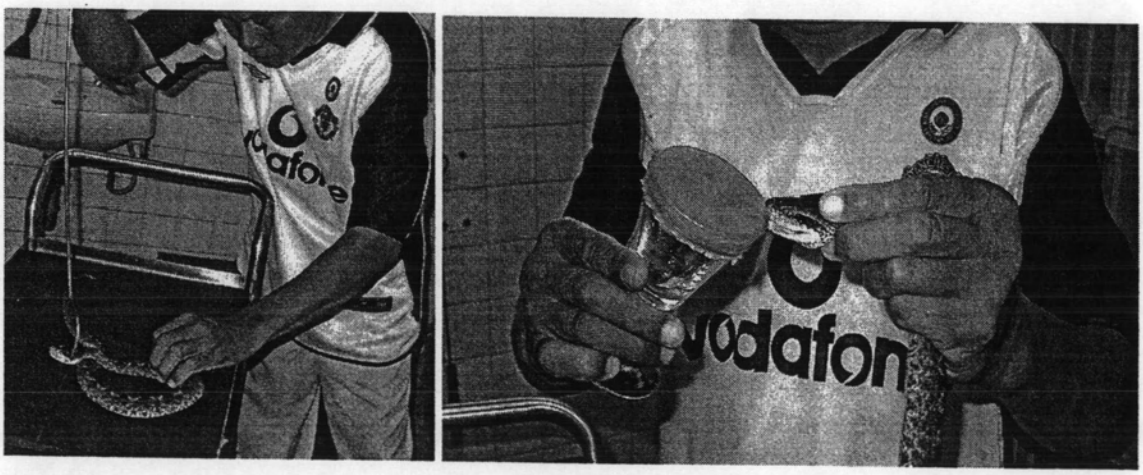

Fig. 3.1 Milking venom.

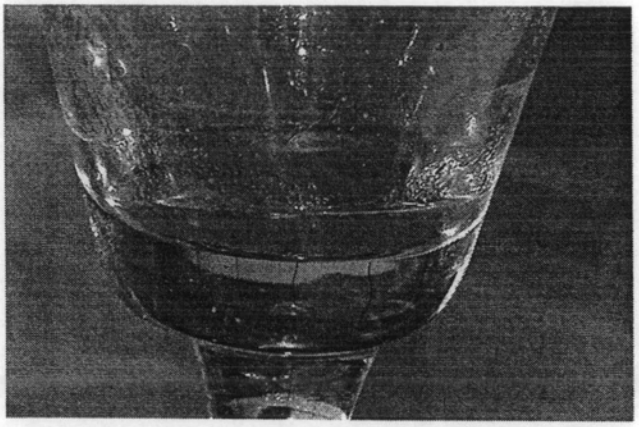

Fig. 3.2 Crude venom

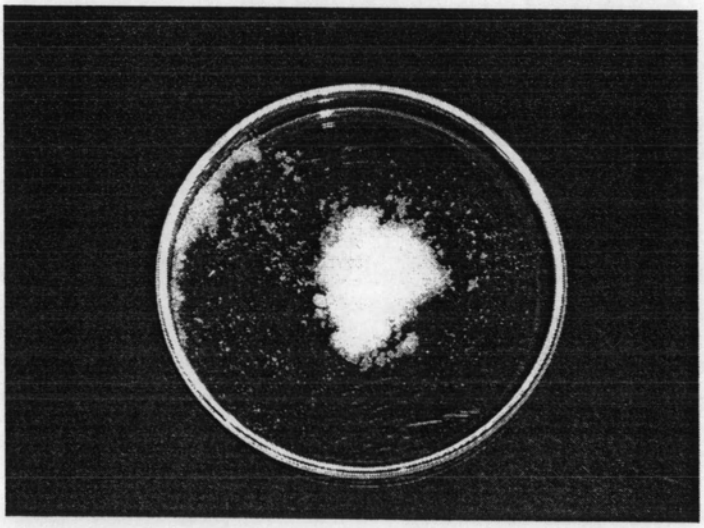

Fig. 3.3 Lyophilized venom

3.3.2 Electrophoretic Titration (ET)

Protein consists of amino acids which have carbonyl of amino group. Protein can have negative, positive or neutral charge depend on the pH. The isoelectric point (pI) is the pH at which a molecule carries neutral charge. At a pH below the pI, proteins carry a net positive charge and proteins have negative charge at pH above pI (Fig. 3.4).

A gradient of 3-9 pH was established on IEF gel using electrophoresis Pharmacia phase system. For 10 min after gradient was established, the gel was rotated degree 90 clockwise. Three µg of 1.5 mg/ml of crude venom was placed on applicator and the applicator was set in the middle of the IEF gel for second dimension. The proteins were developed by silver nitrate staining.

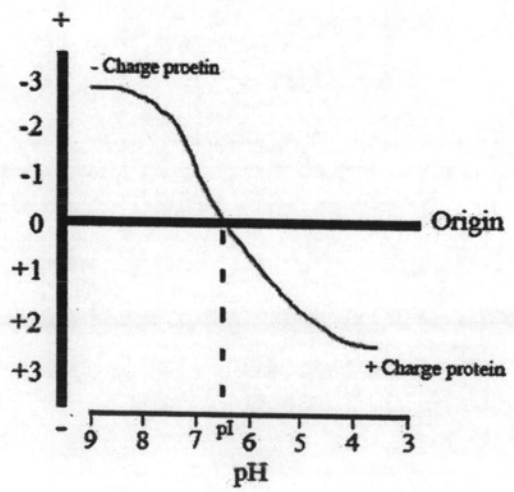

Fig. 3.4 ET profile model

3.3.3 Opossum serum collection

Twenty Virginia opossums (*Didelphis virginiana*) were collect from the area within the Kingsville, TX city limits by Kleberg Country Animal Control officials. The Virginia opossums were anesthetized with ketamine (0.3 ml/kg) and xylazine (0.2 ml/kg), and blood was collected by way of cardiac puncture. The blood was allowed to coagulate for 24 hr at 4 °C. The blood was centrifuged at 10,509 x g for 10 min 4 °C using a Beckman Avanti™ 30 Centrifuge at 4 °C and filtered through a 0.45 um Millex HV filter.

3.3.4 Lethal dose (LD_{50})

Six groups of eight mices for each venom concentration were housed in cages and observed throughout the quarantine period and experiments. Two-fold serial dilutions using saline were made to obtain five additional concentrations. All solutions during the experiment were stored at 4 °C and warmed to 37 °C just before being injected into mice. The lethal toxicity was determined by injecting 0.2 ml of venom (at various concentrations) into the tail veins of 18–20 g female BALB/c mice. The injections were administered using a 1 ml syringe fitted with a 30-gauge, 0.5-in. needle. Saline controls were used. LD_{50} was calculated by the following equation.

$$LogLD_{50} = Log\ (100\%death) - \frac{LogDF\ (\Sigma Die - N/2)}{N}$$

N = Number of mice

Σ Die = Number of mice die

DF =Dilution factor

100%death = Concentrations 100% death (mg/Kg)

3.3.5 Hydrophobic interaction chromatography (HIC)

Crude venom was dissolved by 0.1M sodium phosphate buffer containing 1.8 M ammonium sulfate pH 7.0 (10 mg/ml), centrifuged at 3000 rpm for 5 min and filtrate through by a Acrodisc® 4 0.45μm. A hundred microliters of mixture was injected into Shodex® HIC PH-814 column which equilibrated with buffer A (0.1M sodium phosphate buffer, pH 7.0, containing 1.8 M ammonium sulfate, pH 7.0) elution was then carried out by a linear decrease in the concentration of buffer A, while increase the concentration of buffer B (0.1M sodium phosphate buffer, pH 7.0) gradient over 60 min with a flow rate of 1 ml/min. Absorbance was measured at 280nm. The fractions were dialyzed against Milli-Q water for 12h, then lyophilized, and reconstituted in 0.02M phosphate buffer pH 7.0.

3.3.6 Size exclusion chromatography

Waters protein-pak™ 125 (7.8 x 300 mm) column was connected to a Waters 2487 dual λ absorbance detector. The absorbance was monitored at 280 nm

and flow rate was controlled by Water 1525 binary HPLC pump. The system was equilibrated with 0.02M phosphate buffer, pH 7.0 with a flow rate 0.5 ml/min.

Waters protein-pak™ 60 (7.8 x 300 mm) column was connected to a Waters 2487 dual λ absorbance detector. The absorbance was monitored at 280 nm and flow rate was control by Water 1525 binary HPLC pump. The column was equilibrated with 0.02M phosphate buffer, pH 7.0 with a flow rate 0.5 ml/min. Purity was judged by SDS-polyacrylamide gel electrophoresis.

3.3.7 Reverse phase column

A Waters 2487 dual λ absorbance detector was monitored at 254 nm and control flow rate was controled by Water 1525 binary HPLC pump which was connected to C18 Vydac™ column. The column was equilibrated with 0.01% TFA and run gradient with 80% acetonitrile in 0.01% TFA with 1.0 ml/min flow rate.

3.3.8 Hemorrhagic activity

Hemorrhagic activity is define as the leakage of blood components through the basement membrane. It is caused by proteolytic degradation of the basement membrane protein by the venom hemorrhagic proteinases (Baramova *et al.*, 1989; Bjarnason & Fox, 1994; Bjarnason *et al.*, 1988). A modified hemorrhagic assay described by Omori-Satoh (Omori-Satoh *et al.*, 1972) was used to determine the hemorrhagic activity of crude venom and fraction. A total of 100 µl of each fraction collected from column was injected intracutaniously (i.c.) into the back of New Zealand

rabbit (*Oryctolagus cuniculus*) (Fig 5.5). The rabbit was sacrificed after 18h and the skin removed. The hemorrhagic activity was determined by the appearance of a hemorrhagic spot on the skin of the rabbit. The activity was reported by minimal hemorrhagic dose (MHD).The MHD was defined as the amount of venom protein which caused a 10 mm hemorrhagic spot (Fig 3.6).

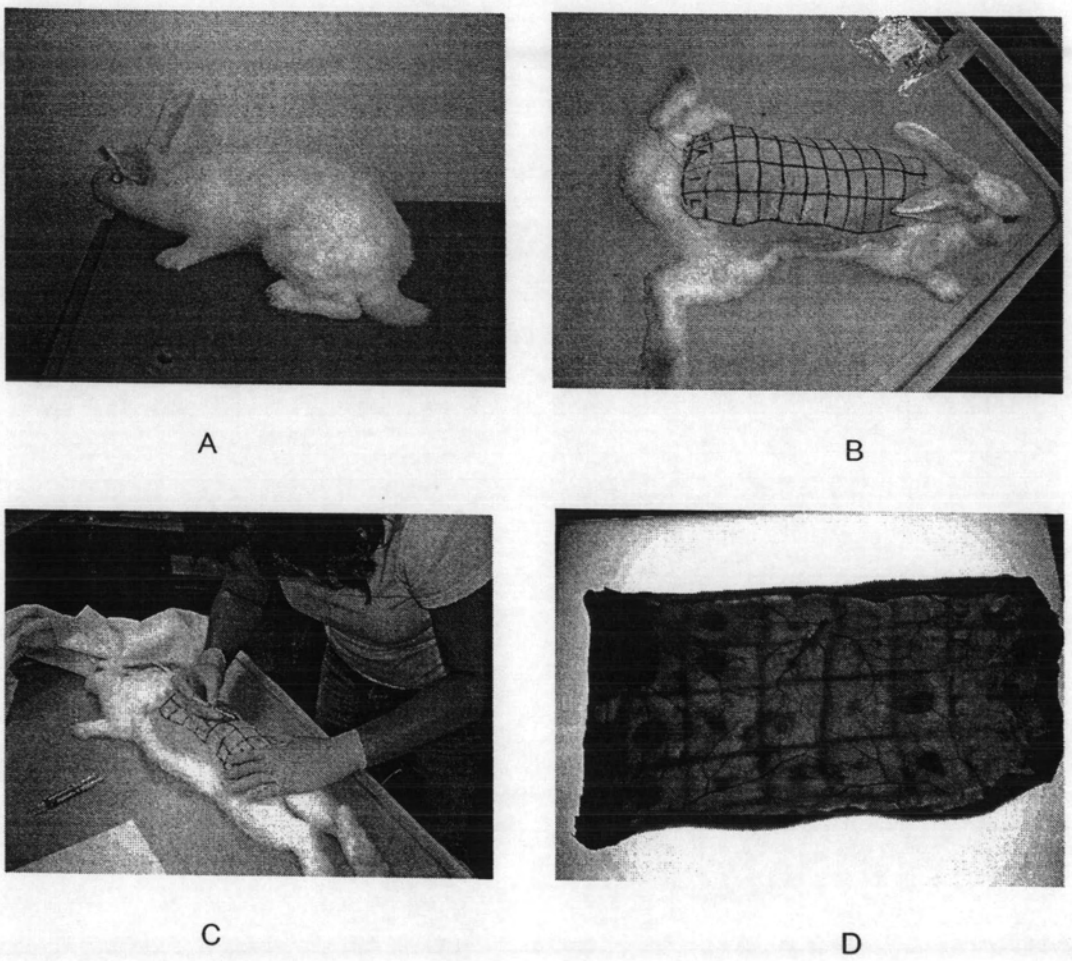

Fig. 3.5 Hemorrhagic test. (A) Rabbit. (B) Prepared rabbit for injection. (C) Injection. (D) Rabbit skin

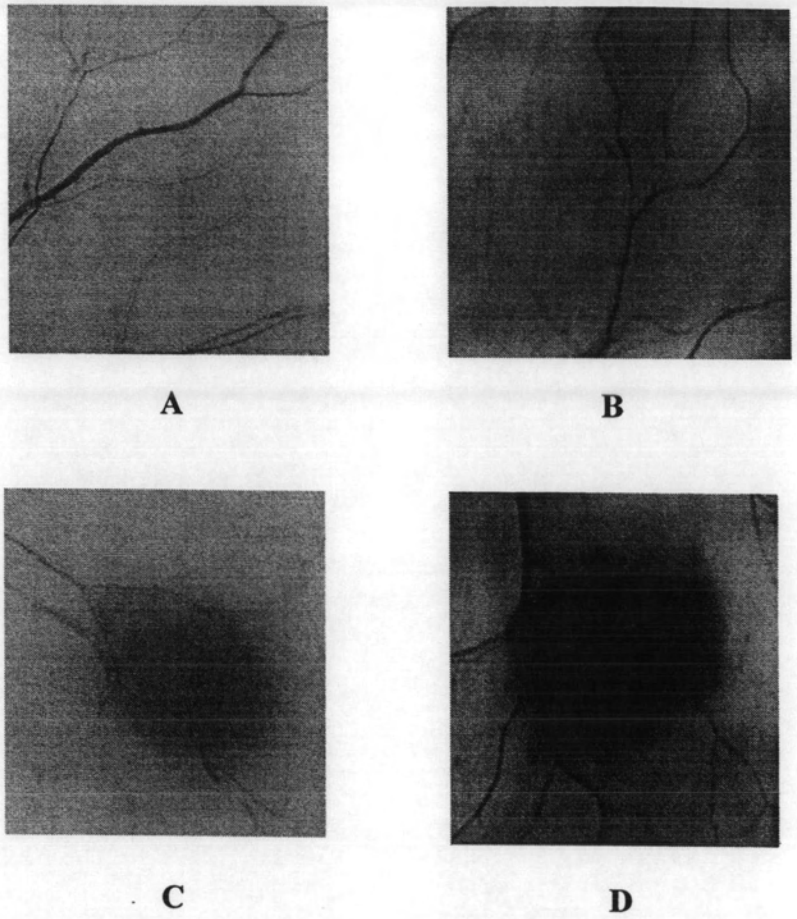

Fig. 3.6 Hemorrhagic activity report (A) no activity (B) weak activity (C) mind activity (D) strong activity

3.3.9 Antihemorrhagic activity

A modified method described by Omori-Satoh (Omori-Satoh *et al.*, 1972) was used to determine the antihemorrhagic activity of *D. virginiana* crude serum against *C. rhodostoma* crude venom. One hundred and fifty microliters of *D. virginiana* crude serum (serial dilutions) was mixed with 150 µL of *C. rhodostoma* 2 MHD and incubated for 1 h at 37°C. The back of an anesthesized New Zealand rabbit (*Oryctolagus*

cuniculus) was depilated and 0.1 mL of the mixture was injected intracutaneously. The rabbit was sacrificed after 24 h. The specific activity (mg) is defined as the concentration of *D. virginiana* sample protein required to reduce 100% of 1 minimal hemorrhagic dose (MHD) times 0.1 mL (volume injected into the back of a rabbit).

3.3.10 Fibrinolytic activity

A method modified from Bajwa (Bajwa *et al.*, 1980) was used to measure fibrinolytic activity of the fractions obtained from *C. rhodostoma* venom. Three hundred microliters of fibrinogen solution (9.5 mg/ml) and 12 µl of thrombin solution (1000U/ml) were added to each well of a 24-well plate. After each well contained both solutions, the plate was shaken gently. The plate was held at room temperature, until well contents become firm and then incubated for 3 h at 37 °C. Ten microliters aliquots from each fractions were added to a separate well of the plate and incubated for an additional 15 h at 37 °C. Seven hundred microliters of 10% trichloroacetic acid (TCA) was placed in each well to stop the reaction, and then decanted off after 10 min. The specific fibrinolytic activity was calculated by dividing the diameter (mm) of the cleared zone in the fibrin by the amount of protein sample (µg) placed in each well. The activity was reported by minimal fibrinolytic dose (MFD).The MFD was defined as the amount of venom protein which caused a 10 mm fibrinolytic zone.

3.3.11 Anti fibrinolytic assay

An antifibrinolytic assay was performed using crude venom. An adaptation of the procedure for determining fibrinolytic activity was used to test for antifibrinolytic activity. The serum of *Didephis virginiama* was used to test the antifibrinolytic activity. An equal volume of serum and venom was incubated for 1 hour at room temperature and 10 µl of this solution was added into each well. The antifibrinolytic activity was measured by the clear area at the bottom of the plate (Perez *et al.*, 2001).

3.3.12 Gelatinase activity

A method modified from Huang and Perez (Huang & Perez, 1980) was used to test gelatinase activity of the venom. Twenty microliters of sample was placed on a Kodak X-OMAT scientific imagining film with gelatinase coating. Hydrolysis of X-ray film was determinated by washing the film with tap water after 4 hours incubation at 37 ^{o}C in moist incubator. The positive samples were perform to determine the clear area on the X-ray film.

3.3.13 Antigelatinase

Equal amounts of 2 MGD of crude venom was mixed with serial dilutions of opossum serum and incubated at room temperature for 1h. Twenty microliters of the sample mixture was place on a Kodak X-OMAT scientific imaging film with gelatinase coating. Hydrolysis of X-ray film was determinated by washing the film with tap water

after 4 h incubation at 37 °C in a moist incubator. The antigelatinase titer is defined as the reciprocal of the highest dilution of animal sera that can neutralize 1 MGD.

3.3.14 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

To determine molecular weight and purity, the isolated fractions were subjected to SDS-PAGE using the Pharmacia phast system™. The sample was mixed with denaturing SDS buffer (10mM Tris-HCl, 2% SDS, 0.1% M DTT, 0.01% bromphenol blue and 1mM EDTA, pH 8.0) at a ratio of 3:1 respectively and boiled for 2 min. Mixture of four microliters of each sample and denaturing buffer mixture were applied to homogenous 12.5 % PhastGel™. Low molecular weight markers for the SDS electrophoresis was used as a reference (Amersham Pharmacia Biotech). The gels was run and developed by silver staining as per manufacturer's directions.

3.3.15 Inhibition of platelet aggregation assay

A Chronolog™ was used to monitor platelet aggregation of all reverse phase chromatography fractions, by impedance, of whole human blood when venom samples were added. Four hundred and fifty microliters of citrated human blood were incubated at 37 °C, for at least 5 min prior to use, with equal amounts of 0.15 M saline solution. Ten microliters of venom fraction was incubated with the blood sample for 2 min. An electrode was inserted in the blood sample, and 90 s later, 20 µL of a 1 mM ADP solution was added to the blood sample to promote platelet aggregation.

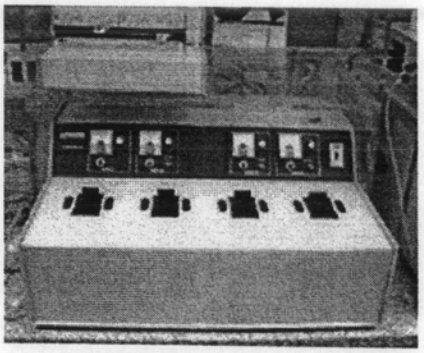

Fig 3.7 Chronolog™ aggregation

3.3.16 Insulin b chain digestion

Bovine insulin b-chain was dissolved in 0.02 M sodium phosphate buffer (1 mg/ml), and two hundred microliters was injected into a C18 reverse phase column. The column was equilibrated with 0.01% TFA and run with gradient of 80% acetonitrile in 0.01% TFA.

Twenty microliters of fibrinogenase enzyme (0.03 mg/ml) was mixed with 180 µL of insulin b-chain solution (1 mg/mL), incubate at 37 °C for 2 h. Two hundred microliters of the mixture solution was injected into a C18 reverse phase column. The column was equilibrated with 0.01% TFA and run with gradient of 80% acetonitrile in 0.01% TFA.

3.3.17 Insulin b-chain digestion inhibitor

Five microliters of *D. virginiana* serum was mixed with twenty microliters of fibrinogenase enzyme (0.03 mg/mL) and incubated at 37 °C for 1 h. One hundred

and eighty µL of b-chain insulin (1 mg/mL) was incubated at 37 °C for 2 h. Two hundred microliters of the mixture solution was injected into a C18 reverse phase column. The column was equilibrated with 0.01% TFA and run with gradient of 80% acetonitrile in 0.01% TFA.

3.3.18 Mass spectrometric analysis

The proteins samples obtained was dried in Vacufuge Eppendorf for 30 min at 30°C and resuspended in 10 µL of 0.1 % TFA/ 50 % ACN, desalted using Zip Tip $_{C18}$ (Millipore ZTC18S096). Then 0.5 µL of α-HCCA (Alpha-cyano-4-Hydroxycinnamic Acid) was spotted on a MTP AnchorChip target plate 600/384 TF (Bruker Daltonics), 0.5 µL of sample was added and mass analysis was performed on the AUTOFLEX II-TOF/TOF (Bruker Daltonics) in positive reflectron mode using external standards: (Insulin Bovine I-5500 Sigma) and (Lysozyme Chicken egg L-6876 Sigma) in order to determine the monoisotopic mass of the intact protein. For protein sequencing analysis, 5 µL of protein was reduced with DTT (Dithio trei-tol), free cysteines were then alkylated using IAA (Iodoacetamide). The reaction was quenched by addition of an excess of DTT, the digestion was made with Trypsin, and the solution was allowed to react overnight at 37°C. The sample was desalted using Zip Tip $_{C18}$. Then 0.5 µL of α-HCCA (Alpha-cyano-4-Hydroxycinnamic Acid) was spotted on a MTP AnchorChip target plate and 0.5 µL of sample was added and mass analysis was performed on the AUTOFLEX II-TOF/TOF (Bruker Daltonics) in positive reflectron mode using external standards:

Peptide Calibration Standard II (Bruker 222570). The protein sequence was determine using MASCOT database.

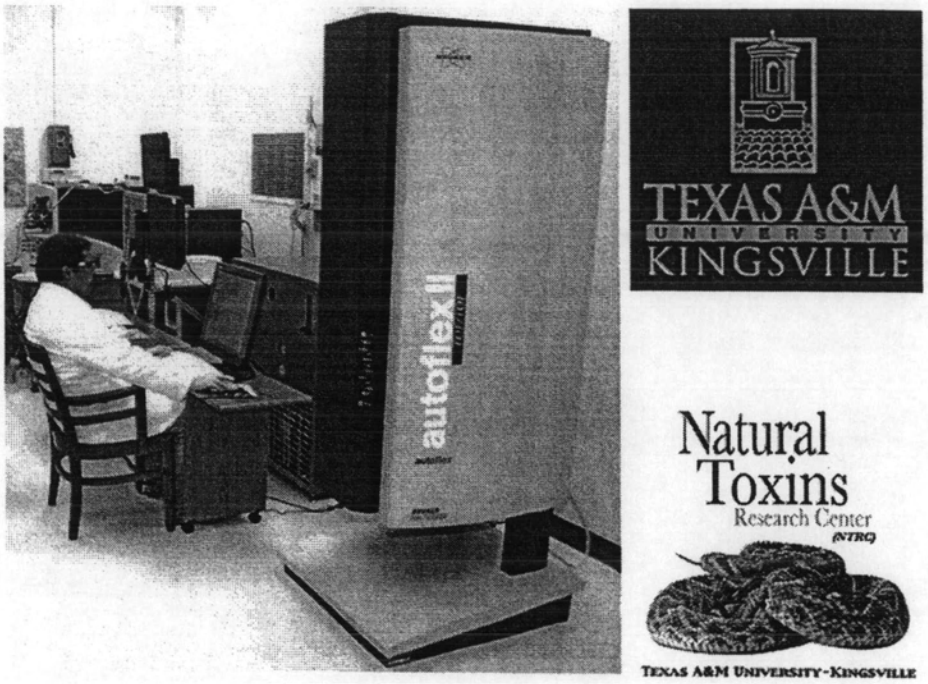

Fig. 3.8 Mass spectrometer, Autoflex II TOF/TOF Bruker Daltonics, Billerica