Chapter III

Methodology

3.1 Materials

3.1.1 Snake Venoms

Dried venoms of green pit viper (T. *popeorum*) and Malayan pit viper (C. *rhodostoma*), using in this study, were obtained by the courtesy of Queen Saovabha Memorial Institute of Thai National Red Cross Society. They were kept in dry state and were freshly made to various dilution with physiologic saline before using.

3.1.2 Fibrinogen

Human fibrinogen was obtained from The Green Cross Cooperation. The concentration were based on an assumed 1 g per bottle. The contents of each concentration used were dissolved with distilled water or specific buffer, adjusted to desired solution.

3.1.3 Thrombin

Bovine thrombin, Thrombostat (Parke-Davis), was used for coagulation work.

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3.2 Methods

3.2.1 DEAE-Cellulose Column Chromatography

The lyophilized venom 300 mg was dissolved in 0.01 N ammonium acetate, pH 7.2, and centrifuged to remove insoluble materials. The venom concentrations used were 3%. A LKB column, 1.6 x 5.0 cm with a bed volume of 250 ml, is packed with DEAE-cellulose (DE 52-Whatman). Before the venom solution is applied, the column was washed overnight with the starting buffer. They are regenerated by washing with 1% sodium hydroxide and equilibrated with 0.05 N ammonium acetate, pH 5.0. Then, the sample is applied onto this column. The chromatograms are developed by a simple gradient elution technique. The elution was initially carried out with 250 ml of 0.5 N ammonium acetate, pH 5.0. Subsequently, the first stage linear gradient from 0.05 N to 1.0 N concentration is achieved by 250 ml of 0.05 N ammonium acetate, pH 5.0, in the mixing vessel and 250 ml of 1 N ammonium acetate, pH 5.0, in the reservior vessel, using as the eluent. The second stage pH gradient elution was carried out with 250 ml of 1 N ammonium acetate, pH 5.0, in the mixing vessel and 250 ml of 1 N ammonium acetate, pH 9.0, in the reservior. The elution was performed at 4° C and the flow rate was adjusted to 25 ml/h and fractions of 5 ml per tube are collected by Fractomette-400 (Searl). The absorbance of eluate fraction was read at 280 nm using

Beckman spectrophotometer, model 25.

Seperated fractions were collected and kept in lyophilized state for further studies.

3.2.2 <u>Determination of Protein</u>

The protein contents of each venom fractions were measured by the method of Lowry et al. (50)

3.2.3 <u>SDS-Polyacrylamide Gel Electrophoresis</u>

The method of Laemmli (1970) was followed with some modification (51), using a 15% polyacrylamide gel as resolving gel, 3% stacking gel, and tris-glycine buffer, pH 8.3, as the electrode buffer. (Appendix I) Venoms and venom fractions are freshly prepared for electrophoresis by dissolving in double distilled water making the concentration of 5 mg/ml, and centrifuged to remove insoluble materials. The non-dissociating buffer containing 40% sucrose, 0.05% bromphenol blue as a tracking dye and 2% SDS. After loading with a 25 µl of sample mixture to each gel well, electrophoresis was then started. It was carried out using non-dissociating discontinous buffer systems under the following experimental conditions: constant voltage, 60 V ; current, 6 mA ; tris-glycine buffer, pH 8.3, as running buffer; and seperations are performed at room temperature until

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the bromphenol blue marker reached the bottom of the gel. The gels after electrophoresis are stained for 1 hour with 0.1% Coomassie brilliant blue R 250 and diffusion-destained by repeated washing in a mixture of methanol-acetic acid-water. (Appendix II)

The character of venom subfraction was expressed as relative mobility (R_f) value. The relative mobility refers to the migration of the protein of interest measured with reference to a tracking dye, where

$$R_f = \frac{\text{Distance migrated by protein}}{\text{Distance migrated by dye}}$$

3.2.4 Thrombin Time

0.3 ml of fibrinogen solution (5 mg/ml) or 0.1 ml of normal pool plasma was incubated at 37°C with 0.1 ml imidazole buffer solution (pH 7.4; imidazole 3.4 g, NaCl 5.85 g, 0.1 N-HCl 186 ml, making up to 1 litre with distilled water). Clotting times were recorded from the addition of 0.1 ml of thrombin or a venom fraction.

The thrombin activity of venom or venom fraction was expressed by the parallel-line assay method, in term relative potency to a reference thrombin of each ditution of 0.1, 0.5, 1.0, 5, 10, 20 and 50 unit/ml, on double logarithmic scale.

3.2.5 <u>Fibrinolytic Activity</u>

The fibrinolytic activity was assessed by lysis of fibrin plate. The modified method of Astrup and Mullertz was employed. (52) The final concentration of fibrinogen was 0.2% in barbitone buffer (pH 7.4; 0.1 M barbitone sodium 570 ml, 0.1 N-HCl 430 ml, NaCl 5.67 g, diluted with an equal volume of 0.9% NaCl before use). Ten ml of the fibrinogen solution were pipetted into each of the 9-cm Petri dishes. The dishes were placed on horizontal surface and the fibrinogen solution was clotted by the addition of 0.3 ml of thrombin (50 N.I.H. unit/ml in 0.9% saline solution) with a micropipette. After shaking the mixture for 20 seconds evenly, a clot was allowed to form. To this clotted surface was then added exactly 25 µl of the test solution. After the plates were incubated for 20 h at 37°C, the lysed zone were measured as the product of the two perpendicular diameters in sq mm (Fig. 1). Tests were set up in duplicate.

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The fibrinolytic activity of venoms and venom fractions were compared in the fashion of the paralell-line assay method with various concentration.

3.2.6 <u>Preparation of Platelet-Rich Plasma</u>

Human blood was withdrawn from a vein and was mixed with 3.8% sodium citrate (9:1 V/V). The platelet-

Figure 1. Fibrinolytic activity by fibrin-lysis plate. Arrows (▲) indicated lysed zone, measured by a x b.

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rich plasma (PRP) was obtained by centrifugation at 1000 rpm at room temperature for 10 minutes. The plasma samples are removed and placed in plastic tubes. The remanining blood was then recentrifuged at 3000 rpm for 15 min and the platelet-poor plasma (PPP) was obtained. Platelet count of PRP was adjusted with PPP to 200-400 x 10^9/l.

Platelet preparations were used with 2 hours after blood withdrawal.

3.2.7 Platelet aggregation Test

This was measured by the turbidimetric method (53, 54), using a dual channel aggregometer (Model ELVI 840). PRP 0.4 ml was pre-warmed at 37°C for 2 min in plastic cuvette, and then the batteries of platelet aggregating inducers: ADP 5 µM, adrenaline 0.1 mg/ml, thrombin 0.25 unit/ml, and collagen 100 µg/ml were added. Platelet aggregation was monitored by continuously recording light transmission in an aggregometer with constant stirring at 1000 rev/min, the aggregation curve was observed for up to 3 min. The percent aggregation was expressed as follows:

$$\text{Aggregation (\%)} = \frac{\text{T after aggregation} - \text{initial T}}{\text{T of PPP} - \text{initial T}} \times 100$$

where T is the light transmission.

3.2.8 Direct Platelet Aggregation

The method was similar to platelet aggregation test. To 0.4 ml of PRP, 0.04 ml of 0.01 M phosphate buffer saline (pH 7.4) or a given dilution of venom or venom fraction, making the final concentration of venom to be 1, 5, 10, 20, and 50 µg/ml, was added without other platelet aggregating inducers. The aggregation pattern and percent aggregation were recorded.

3.2.9 Platelet Aggregation Inhibition Test

As platelet aggregation test, 0.025 ml of the venom or venom fraction or phophate buffer saline was added 3 min prior to the addition of a platelet aggregating inducer. The final concentrations of the venom were 1, 5, 10, 20 and 50 µg/ml.

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3.2.10 Assay of Hemorrhagic Activity

The hemorrhagic activity was assayed by the method of Kondo et al. (55) Test solutions, venom or venom fraction in a volume of 0.1 ml in 0.9% saline, were injected intracutaneously into the depilated back skin of albino rabbit weighing about 3 kg. The injection sites were spaced at distances of about 2.5 cm. Saline solution without venom was also injected as a control. After 24 hours, the rabbit was sacrificed by chloroform

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inhalation and the skin was removed immediately. The intensity of skin response was estimated from the visceral side. The skin was spread and fixed on a glass plate so as to keep the original size. The cross-diameters of each hemorrhagic spots were measured and the mean of the 2 diameters was taken as an indicators of the intensity of the response.

Two rabbits were used under the above described conditions. The log dose-response curves, plotted between log doses of venom and the size of hemorrhagic spots, were established.

The minimum hemorrhagic dose (MHD) of venom was defined as the least quantity of venom, which produced a hemorrhagic spot of 10 mm in diameter 24 hours after intracutaneous injection under the above conditions.

The specific activity of hemorrhagic activity was expressed by MHD per μg protein.

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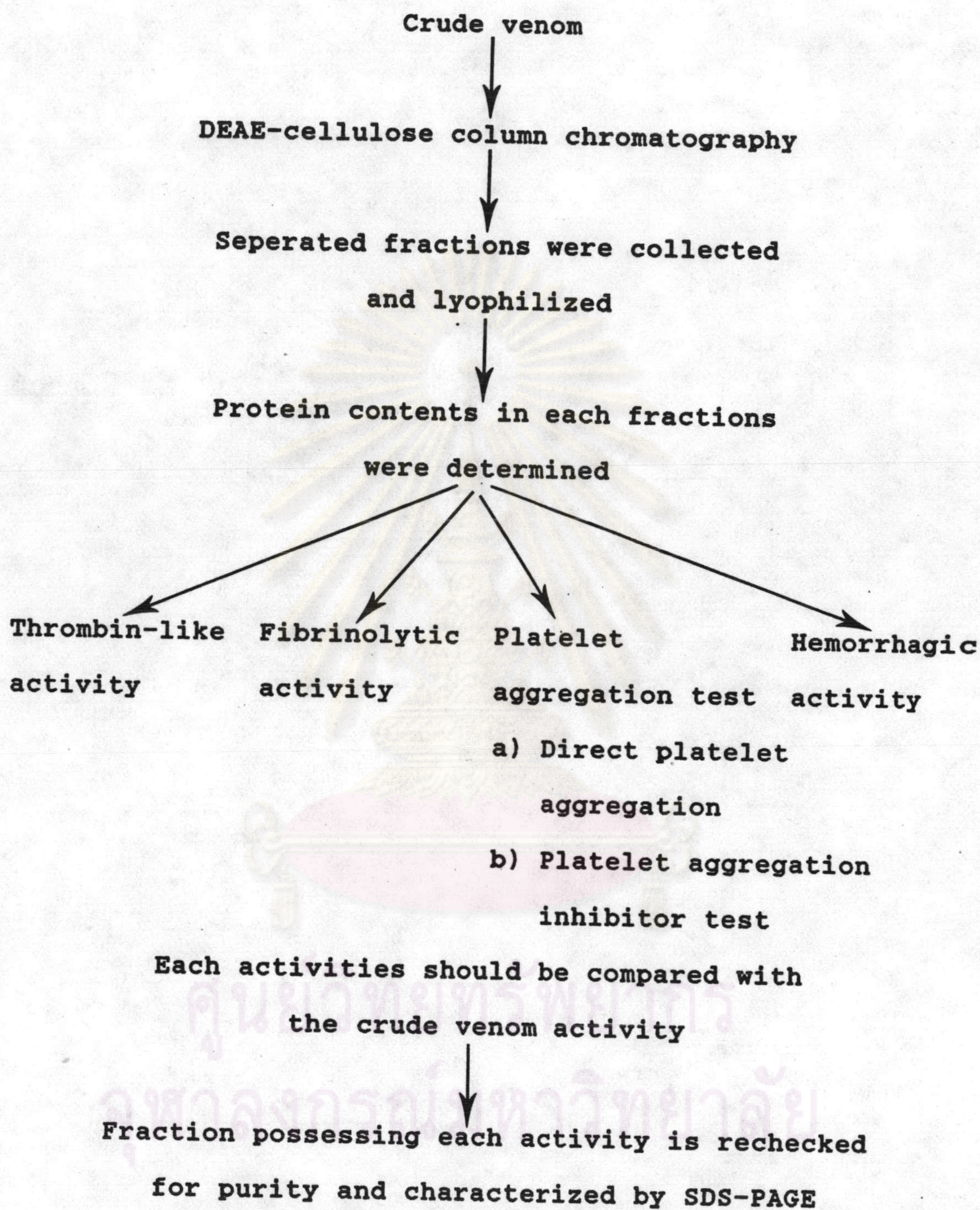

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Figure 2. Architecture of the Study