pH gradient electrophoresis and biological activity analysis of proteins from Malayan pit viper (*Calloselasma rhodostoma*) venom

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ABSTRACT: The Malayan pit viper (*Calloselasma rhodostoma*) is a snake found in most of Southeast Asia. The snake's venom contains proteins with various biological effects. In this study, proteins from Malayan pit viper venom were analysed by electrophoresis titration (ET) and two dimensional gel electrophoresis (2-D gel). In addition, venom proteins were separated by high performance liquid chromatography (HPLC) connected to a hydrophobic interactive chromatography (HIC) column. Fractions collected from HPLC were tested for biological activities. As the result, the ET profile showed that crude venom consisted of both positively and negatively charged proteins. Most of the 191 protein spots found on 2-D gel of crude venom have an isoelectric point in the range 4.5–5.5. After HPLC, eighteen fractions were eluted from HIC column. Each fraction was tested for fibrinolytic, haemorrhagic, gelatinase, and disintegrin activities. Both fibrinolytic and haemorrhagic fractions showed gelatinase activity as well, while the fibrinolytic fraction had no haemorrhagic activity. Our results are valuable to venom research and drug discovery.

KEYWORDS: snake venom, Malayan pit viper, *Calloselasma rhodostoma*, 2-D gel, ET profile

INTRODUCTION

Malayan pit viper (*Calloselasma rhodostoma*) is a common snake found in Southeast Asia (Fig. 1). Human invasion and city expansion into the natural habitat of the snake increases the risk of venomous snakebites[1–3]. Snake venoms are complex mixtures of biological molecules, whose components can be classified into several families such as serine proteinase, metalloproteinase, C-type lectin, disintegrin, and phospholipase[4–6]. The biological effects of these proteins are coagulation, anticoagulation, platelet-activation, anti-platelet aggregation, fibrinolytic, and haemorrhagic activity[7,8]. Some venom components such as disintegrin and fibrinolytic have been extensively investigated as they have the potential for use as therapeutic agents for cancer and cardio- or cerebro-vascular disorders[7–15].

The aim of this study was to analyse proteins in the venom of the Malayan pit viper using electrophoresis titration (ET), two dimensional gel electrophoresis (2-D gel), and high performance liquid chromatography (HPLC) with a hydrophobic interactive chromatography (HIC) column. Fibrinolytic, haemor-

Fig. 1 Geographical distribution of *C. rhodostoma*.

rhagic, gelatinase, and disintegrin activities were also determined. Some of the venom proteins are toxic but some of them have fibrinolytic and disintegrin activities which could be developed as therapeutic agents. This information is valuable for snake venom research including antivenom, drug discovery, and protein development.

MATERIALS AND METHODS

Crude venom

Venom of Malayan pit viper (*C. rhodostoma*) was extracted from snakes maintained at the Queen Saovabha Memorial Institute Serpentarium, Thai Red Cross Society, Bangkok. Venom was collected through the biting of the snake on a nylon cloth membrane covering a venom collection vessel. Crude venom was then pooled and lyophilized.

Electrophoresis titration

The profile and isoelectric point (pI) of venom proteins were determined by ET (Pharmacia Biotech PhastSystem). A polyacrylamide isoelectric focusing (IEF) PhastGel with pH gradient 3–9 was first activated. Then the gel was rotated 90° and a 3.5 µl (1 mg/ml) venom sample were applied. After the electrophoretic step, the gel was automatically stained with silver nitrate.

Two dimensional gel electrophoresis

IEF was the first dimension of 2-D gel and was carried out on 13-cm immobilized pH gradient (IPG) strips with the IPGphor system, Amersham Biosciences. The IPG strips were rehydrated under a silicone oil covering with rehydration buffer containig 50 µg of embryo protein or yolk sac membrane lysate for at least 12 h at 20 °C. Before starting the IEF, damped paper bridges (3 mm wide) were placed over both ends of the IPG strip followed by the electrodes over the paper bridges at each end. Electrophoresis was carrried out as follows: 500 V for 1 h followed by gradient at 1 kV for 1 h and gradient at 8 kV for 2.5 h, with the current limit of 50 µA per IPG strip. After IEF, the IPG strips were equilibrated with 5 ml of SDS equilibration buffer consisting of 0.05 M Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 0.025% (w/v) bromophenol blue. Briefly, the IPG strips were placed in a tube containing equilibration buffer with dithiothreitol (50 mg/5 ml). After shaking for 20 min, the strips were placed in a tube containing equilibration buffer with iodoacetamide (125 mg/5 ml) and was shaken for 20 min. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) without stacking gel was used as the second dimension. Equilibrated IPG strips were applied on top of polyacrylamide gels (12.5% T, 2.6% C, 14 × 6 cm) and sealed with 0.5% agarose in electrode buffer. Electrophoresis was carried out using constant current of 10 mA per gel for 15 min, followed by 20 mA per gel until the dye reached the front edge of the gel. The gel was stained with Brilliant Blue. After destaining, the gel was scanned and analysed with the IMAGE MASTER 2D Elite analysis software.

Hydrophobic interactive chromatography

Crude, lyophilized *C. rhodostoma* venom was dissolved to a concentration of 10 mg/ml in 0.1 M sodium phosphate buffer containing 1.8 M ammonium sulphate pH 7.0, centrifuged at 3,000 rpm for 5 min and filtrated by an Acrodisc (0.45µm). One milligram of crude venom was injected into Shodex HIC PH-814 column pre-equilibrated with buffer A (0.1 M sodium phosphate buffer, pH 7.0, containing 1.8 M ammonium sulphate). Elution was carried out by a linear decrease in the concentration of buffer A and an increase in the concentration of buffer B (0.1M sodium phosphate buffer, pH 7.0) for 60 min at a flow rate of 1 ml/min. Proteins were monitored by measurement of absorbance at 280 nm. The fractions collected were dialysed against Milli-Q water for 12 h, lyophilized, and then reconstituted in 0.02 M phosphate buffer pH 7.0. The fractions were tested for haemorrhagic, fibrinolytic, gelatinase, and disintegrin activities.

Haemorrhagic activity assay

A modified haemorrhagic assay described by Omori-Satoh[16] was used to determine the haemorrhagic activity of *C. rhodostoma* venom. A 100 µl sample was injected intracutaneously (i.c.) into the back of a New Zealand white rabbit (*Oryctolagus cuniculus*). The rabbit was sacrificed after 18 h and the skin was removed. The haemorrhagic activity was determined by the boundary of a haemorrhagic spot on the skin of the rabbit.

Fibrinolytic activity assay

A modified method from Bajwa[17] was used to measure fibrinolytic activity of *C. rhodostoma* venom. First, 300 µl of fibrinogen solution (9.5 mg/ml) and 12 µl of thrombin solution (1,000 U/ml) were added to each well of a 24-well plate. The plate was shaken gently at room temperature until the mixture contents became firm and then the incubation was continued for 3 h at 37 °C. Then, 10 µl of sample were added to each well and the plate was incubated for additional 15 h at 37 °C. To stop the reaction, 700 µl of 10% trichloroacetic acid (TCA) were added to each well and decanted off after 10 min. The clear zone in the fibrin was observed and its diameter was measured.

Gelatinase activity assay

Gelatinase activity of the venom was tested using a modified method of Huang and Pérez[18]. Samples (20 µl) were placed on a piece of gelatine coated Kodak X-OMAT scientific imaging film. After 4 h incubation at 37 °C in a moist incubator, hydrolysis of the gelatine on the film was terminated by washing the film with tap water. Gelatinase activity was exhibited by a transparent spot on the X-ray film and its diameter was measured.
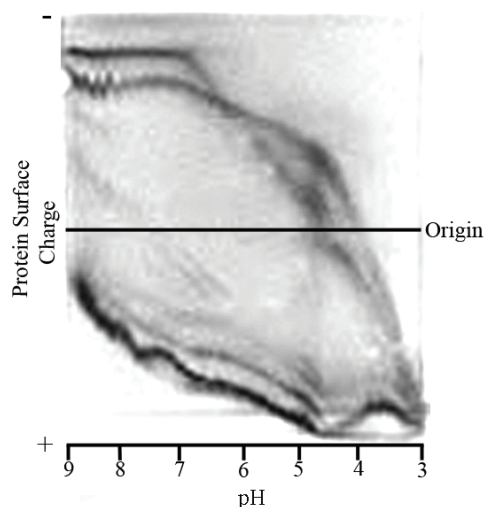
Inhibition of platelet aggregation assay

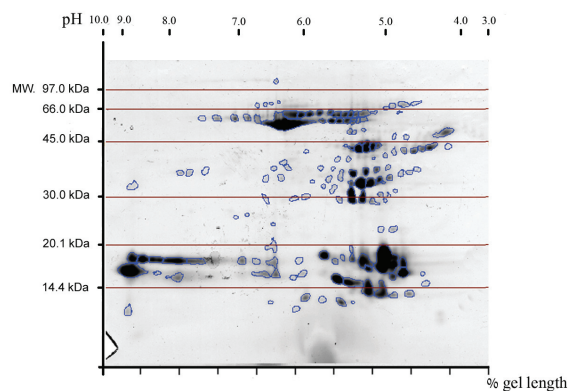A Chronolog Lumi-Aggregometer was used to

monitor platelet aggregation. Citrated human blood (450 µl) was incubated at 37 ºC for at least 5 min prior to use, with equal amounts of 0.15 M saline solution. The venom fraction (10 µl) was incubated with the blood sample for 2 min. An electrode was inserted in the blood sample and 1 mM ADP solution (20 µl) was added to the blood sample 90 s later to promote platelet aggregation.

RESULTS

Crude venom contains both basic and acidic proteins, as indicated by the ET profiles (Fig. 2). The isoelectric point (pI) range for all proteins was 3–9. This can be observed on the x-axis at the origin and differences in the surface charge are viewed on the y-axis. The venom proteins profile of the 2-D gel is


Fig. 3 Two dimensional gel electrophoresis (2-D gel) profile of *C. rhodostoma* venom.

shown in Fig. 3. The pI range 3–10 can be observed on the x-axis. The molecular weight of the proteins was 14–97 kDa, as can be seen on the y-axis. The venom was found to contain 191 proteins. Eighteen fractions were collected from the HIC. The fibrinolytic fractions are shaded and the ET profile is shown in the upper right corner of the HPLC chromatogram (Fig. 4). Those proteins are positively charged. The hydrophobic interaction force of all proteins can be observed on the x-axis. Each fraction was tested for fibrinolytic, haemorrhagic, gelatinase, and disintegrin activities. The results are shown in Table 1. Fraction number six showed potent fibrinolytic activity, but no haemorrhagic activity. However, fractions with both fibrinolytic and haemorrhagic activities also possessed gelatinase activity.

Fig. 2 Electrophoresis titration (ET) profile of *C. rhodostoma* venom.
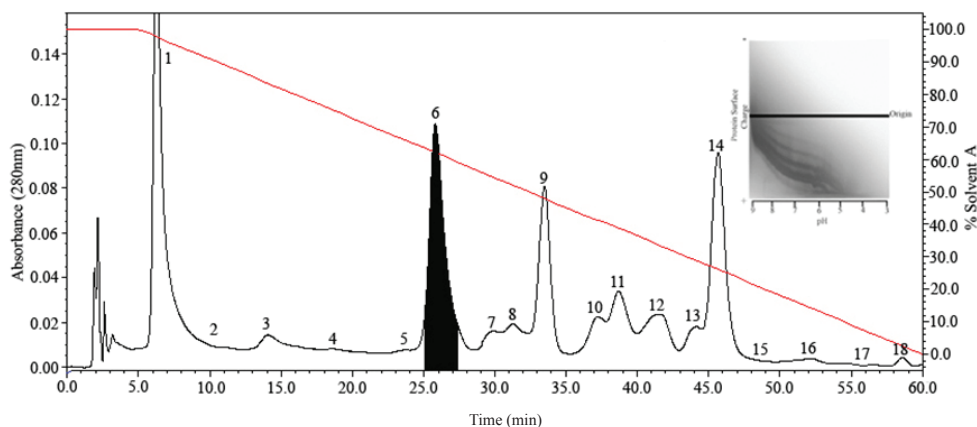
Fig. 4 Chromatogram from hydrophobic interactive chromatography (HIC) column. The shaded area indicates the fractions with potent fibrinolytic activity. The ET profile of this fraction is shown in the upper right corner.

Table 1 Biological properties of fractions from HIC column.

Fractions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Activities																		
Haemorrhagic(mm)[a]	-	1	-	2	2	-	-	-	-	2	10	2	-	-	-	-	-	-
Fibrinolytic (mm)[a]	-	-	-	-	-	10	3	2	1	-	-	-	-	-	-	-	-	-
Gelatinase (mm)[a]	-	2	-	2	-	8	-	-	-	2	10	2	-	-	-	-	-	-
Disintegrin	-	-	-	-	P[b]	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Defined as a diameter of lysed zone
[b] Positive

DISCUSSION

ET is an easy and useful technique to determine the pI of protein. The titration curve shows a banding pattern which represents the surface charge of the protein. The pH differences in the gel alters the surface charge of venom proteins. This surface information can be used to predict the optimal condition to separate venom via ion exchange techniques. As the pH decreases below the pI, the protein surface becomes more positively charged and venom proteins migrate towards the cathode. In contrast, if the pH increases above the pI of the protein, the surface becomes more negatively charged and the venom proteins migrate towards the anode. 2-D gel is an advanced technique to identify protein by separating them according to two variables.

HIC separates proteins according to their hydrophobic properties. The hydrophobic groups on the protein surface bind to hydrophobic groups on the column. The more hydrophobic the protein is, the stronger it binds to the column. Ammonium sulphate increases the hydrophobic interaction and therefore the protein can be eluted by decreasing the concentration of ammonium sulphate. In addition, it also stabilizes proteins so that proteins separated by an HIC column are in the most stable form.

Malayan pit viper contains haemorrhagic toxin[19–21]. Protein with haemorrhagic activity was separated from other proteins. Our results confirm that the protein fractions with fibrinolytic activity are not those containing haemorrhagic activity. Recently, fibrinolytic and disintegrin activities have been developed as therapeutic agents. In the future, venom protein with therapeutic potential could be modified for human use.

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