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CRUDE RUSSELL'S VIPER VENOM A SUPERIOR PROCOAGULANT COMPARED TO ITS ISOLATED, PURIFIED AND CHARACTERIZED TOXIN

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ABSTRACT
Whatman41Paper electrophoresis employing Barbital buffer pH (9.0) at 300 volts for 3 hours separated the Vipera russelli (Indian Russell's viper) venom in its basic components. This lead preliminary experiment helped in the selection of an appropriate HPLC (Shim Pack CLC-ODS (M)) column in a reverse operation/separation mode using 75% Acetonitrile as an eluent. Subsequent reverse phase preparative HPLC fractionated, the crude Russell's viper venom into 10peaks, whose protein contents were quantitatively determined by the FCR method. Peak which showed the highest protein concentration was further subjected to SDS-PAGE electrophoresis, molecular weight determination and procoagulant activity profile.

KEY WORDS: Snake venom, Electrophoresis, Drug-design, Procoagulant activity.

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INTRODUCTION

RWP Master and S. Srinivas Rao reported the electrophoretic identification of enzymes and toxins in the venoms of Indian Cobra and Indian Russell's viper on starch gel in 1961[1]. Snake venoms are a mixture of a group of enzymes viz., Phospholipase A_2, Protease, Phosphomonoesterase, Hyaluronidase, Acetylcholineesterase, 5'Nucleotidase, and toxins of biological and therapeutic interest[2].

Blood coagulation system is an extremely complicated molecular physiological phenomena comprising of two pathways viz; intrinsic and extrinsic pathway and 13 blood coagulation factors acting in a directed manner. The blood coagulation process can also be regarded as a science of system of control and communication particularly in living animals. Some of the controlling components are the positive and negative feedbacks, multiple enzyme participation, 1:1 reactions and a close interaction with organ function. The blood coagulation process can be divided into three basic events viz; (i) Formation of factor X a (ii) Formation of thrombin, (iii) Formation of fibrin.

It is very important that in the event of an injury or accident or a disorder blood has to clot in particular time slot (The normal clotting time for a healthy individual weighing 70kgs is (5-15minutes) and therefore, if the clotting time is prolonged there would be copious loss of blood resulting in the drop of systolic blood pressure, followed by circulatory collapse, ultimately resulting in a state of shock and heart failure .Hence, such severe loss of blood should be prevented and if natural physiological coagulation cannot be brought about due to reasons like genetic disorders like hemophilic and hemorrhagic syndromes, circulating anticoagulants, thrombocytopenic purpuras, dental extractions, prostractomy, Ophthalmological surgeries, gastroenterology, cosmetic surgeries and post delivery bleedings[3]. One must make use of therapeutic external procoagulant support to hasten the blood coagulation process.

In the present investigation, Russell's viper crude venom was separated into its components by a paper electrophoretic technique and their basic nature was confirmed. This was followed by reverse phase high performance liquid chromatography (RPHPLC) technique and the components thus obtained were analyzed for their protein content. The lyophilized component of interest was further subjected to SDS PAGE electrophoresis, where a single band confirmed its purity. Further, the same component's molecular weight was determined, and its procoagulant capability was studied and compared with that of crude Russell's viper venom.

MATERIALS

Crude Russell's viper venom, was procured from The Irula Snake catcher's Society, Chennai, India (30mg of Vipera russelli crude venom in 1ml of distilled water for HPLC), 10% Russell's viper Venom in distilled water (for paper electrophoresis) Paper electrophoresis apparatus, Whatmann41, filter paper, Barbital buffer pH 9.0, 0.1% Bromophenol blue dye in methanol for staining, 5%Acetic acid solution for destaining, HPLC unit (Shimadzu Shim Pack (CLC-ODS(M), Acetonitrile (eluent), Gel electrophoresis unit, Standard molecular weight markers (Bangalore Genie), Power pack unit for gel electrophoresis, Acrylamide, Bis Acrylamide, Sodium dodesylsulfate (SDS), Tris glycine buffer pH 8.8, Tris glycine buffer pH 6.8, Tank buffer (1x concentration) Coomassie brilliant blue dye for staining the gel Ammonium per sulfate, TEMED, Sodium chloride , egg lecithin, Tris hydrochloric acid buffer , Human plasma, Calcium chloride.

METHODS

Barbital Buffer (0.2Molar, pH 9.0) preparation

Stock solution A: - 0.2 Molar sodium barbital solution (41.2 gm/liter)

Stock solution B: - 0.2 Molar HCl Solution (18ml of conc. HCl per liter of distilled water)

Working buffer: 50 ml of stock solution A + 2.5ml of stock B made to 200ml with distilled water pH of the buffer was adjusted to 9.0 Whatman 41 paper was cut into 50cm long and 2.5cm wide strips .

100µl of Vipera russelli crude venom was applied in the centre of the strip(↑) with a micro pipette and dried in air and then subjected to electrophoresis in a Barbital buffer system (pH 9.0) at 300volts for 3hours.After the electrophoretic run ,the strips were dried overnight in air, at room temperature, rolled

and stained with bromophenol blue dye for one hour, and subsequently destained with 5% acetic acid till the separated components were clearly visible[4]. 30mg of crude Vipera russelli venom were dissolved in one ml of distilled water, and 15µl was subjected to reverse phase HPLC analysis on Shimadzu (Shim-Pack) system and the separated crude venom components were eluted with Acetonitrile[5-7]. The fraction with the highest protein concentration was subjected to SDS- PAGE and its molecular weight was determined [8-10]. procoagulant activity was determined by the method of W.J Williams and M.P.Esnouf [11] in which 0.1 ml of fresh human plasma was incubated with 0.1 ml of crude Russell's viper venom in 0.01M Tris-saline buffer pH 7.3,and 0.1ml of phospholipid emulsion in 0.01M Tris-hydrochloric acid buffer , pH 7.3 to this mixture 0.1ml of buffered 0.05M calcium chloride was added and clotting time was recorded in seconds. Similar analogy was employed for the isolated fraction.

RESULTS

Movement of the crude Russell's viper venom components towards the negative electrode in the paper electrophoretic experiment clearly demonstrated its basic nature **(Fig 1 and table1).** Taking a clue from this experiment, Vipera russelli venom when subjected to preparative reverse phase HPLC separation got clearly separated into 10 peaks **(Fig 2).** The fraction with highest protein concentration but a low molecular weight of approximately 6.9 K Da is the Russell's viper pure toxin. **(Fig 3)**. Blood coagulation results for Crude Vipera russelli venom. 5µl of crude Russell's viper venom brings about clot formation in 8seconds where as 5µl of isolated and purified fraction brought about the clot formation in 11 seconds.

DISCUSSION

Isolation of a venom component or a factor in its pure form by Reverse phase HPLC technique could act either as a potential drug or a chemotherapeutic agent or as a guide to synthesize an active drug molecule. Hence could play a vital role in drug design and development. Comparing the procoagulation time data of the crude Russell's viper venom and that of the reverse phase high performance liquid chromatography isolated pure Russell's viper venom toxin definitely confirms the superiority of the crude Russell's viper venom over that of the isolated and purified 6.9KDa toxin which could be in turn attributed to other factors in the crude venom viz; enzymes like Phospholipase A_2, Protease, Phosphomonoesterase, Phosphodiesterase, Hyaluronidase, Acetylcholineesterase, 5 Nucleotidase probably exerting their synergistic effect in bringing about a faster procoagulant effect compared to its purified toxin.

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Table 1: **Paper electrophoresis pattern of the venom of Vipera russelli (Indian Russell's viper)**

Venom	System	pH	Molarity	Anodic components	Cathodic components	Total
Vipera russelli	Barbital buffer	9.0	0.2	----	04	04

Table 2: Protein estimation of different fractions of *Vipera russelli by* FCR method

Fraction Numbers	Concentration of protein in µg
Fraction 1	20
Fraction 3	40
Fraction 4	60
Fraction5	68
Fraction 6	65

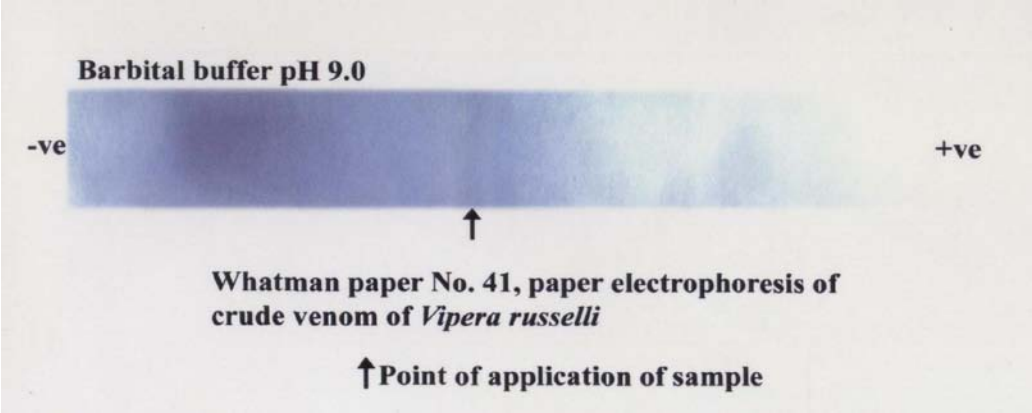


Figure. 1

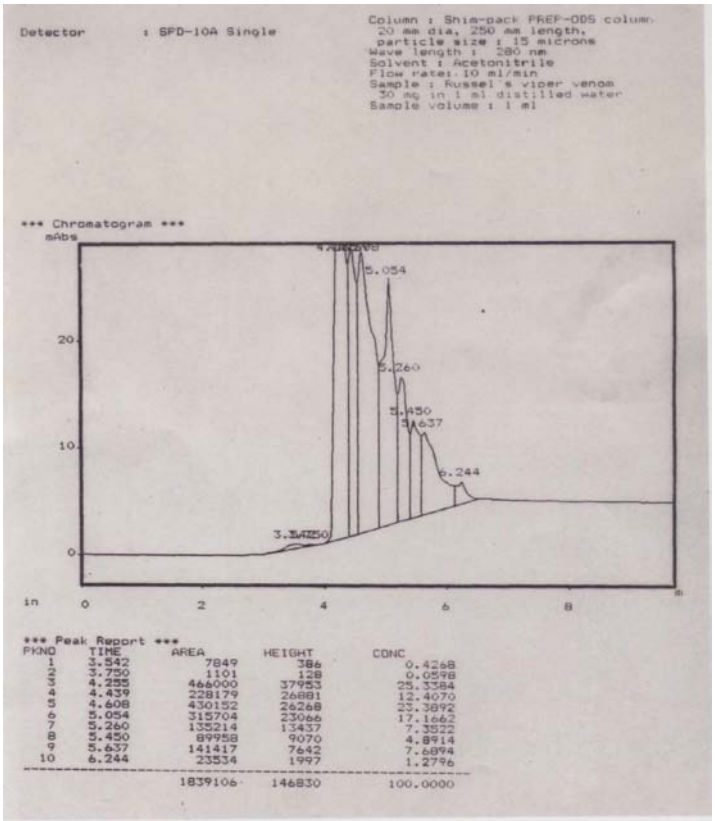
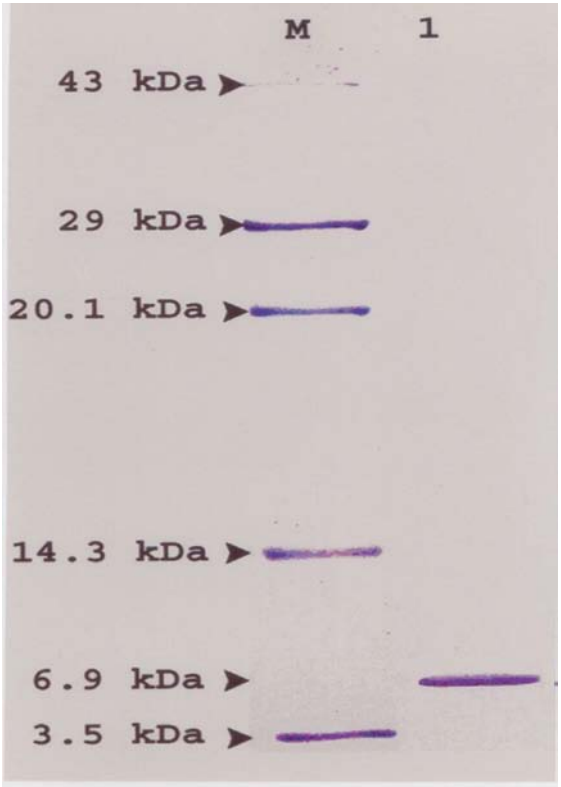


Figure 2 Graph showing fractions of preparative RPHPLC of *Vipera russelli* crude venom

Figure 2: Graph showing the fractions of preparative RPHPLC of Vipera russelli crude venom



SDS-PAGE of *Vipera russelli* venom fraction 5
M- Standard molecular weight marker
Lane1- Fraction no. 5

Figure 3: SDS-PAGE of Vipera russelli venom fraction 5