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SNAKE VENOM:

THEIR PURIFICATION AND ISOLATION AND ANALYSIS OF THEIR ANTI-VENOM ANTICOAGULANT PROPERTIES

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ABSTRACT… Introduction: Viperidae venoms contain toxins that are direct or indirect anticoagulants that inhibit the clotting pathway, therefore increasing the risk of bleeding. Several venoms from the families Viperidae contain proteolytic enzymes that exercise some effect on the blood coagulation process. Snake venom toxins which delay blood coagulation are proteins or glycoprotein with molecular weights ranging from 6 kDa to 350 kDa. These factors inhibit blood coagulation by different mechanisms.Some snake venoms contain toxins that are direct or indirect anticoagulants, which inhibit the clotting process, thus increasing the risk of bleeding. Snake venom toxins that prolong blood coagulation are proteins or glycoproteins with molecular masses ranging from 6 to 350 kDa. **Methods:** The crude venom of E. carinatuswas obtained from the Venomous Animals from department of microbiology Hazara University, Mansehra (Pakistan). Sephadex G-75 and DEAE-Sepharose columns were purchased from Pharmacia (Sweden). CaCl2 and PT kit was purchased from Fisher Diagnostics (USA). Protein markers were obtained from BioRad (Hercules, USA). Other reagents and chemicals were of analytical grade from Fluka and Merck. **Results:** The anticoagulant fractions (F2C and F2D) isolated in the present work were characterized as proteases, since a photolytic effect was observed on casein, BAPNA or human plasma. Our results showed that the PT value significantly increased in the F2C and F2D fractions as compared with PT value of the crude venom. **Conclusions:** Inthe present study, the venom of Echiscarinatuswas fractionated by chromatography and each fraction evaluated by PT test. These fractions showed enzymatic activity. Their main components were proteins of molecular weights of about 42, 50 and 79 kDa. . Further studies are needed to verify this hypothesis.

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INTRODUCTION

Viperidae venoms contain toxins that are direct or indirect anticoagulants that inhibit the clotting pathway, therefore increasing the risk of bleeding. Clinically, this may be little different in effect than the consumptive route used by procoagulants, although, in general, it can be concluded that the anticoagulant venoms are associated with less severe pathologic bleeding than consumptive venoms[1].

Several venoms from the families Viperidae contain proteolytic enzymes that exercise some effect on the blood coagulation process. Snake venom toxins which delay blood coagulation are proteins or glycoproteins with molecular weights ranging from 6 kDa to 350 kDa. These factors inhibit blood coagulation by different mechanisms[2].

An anticoagulant activity has been reported from different snake venoms and their responsible proteins have been purified. Some reports indicate that the anticoagulant action of snake venom proteins is attributed to: (a) activation of protein C, (b) inhibition of blood coagulation factors IX and X by a venom protein that binds to either or both

clotting proteins, (c) a thrombin inhibitor and (d) phospho-lipases that degrade phospholipids involved in the formation of complexes critical to the activation of the coagulation pathway. The anticoagulants isolated from snake venoms prolong clot formation; they are enzymes, such as serine and metalloproteases, or nonenzymatic proteins, such as C-type lectin-related proteins and three-finger toxins[3,4,5].

In the present study the venom of snake Echis carinatus (IEc) was fractionated by chromatography and the anticoagulant effect of each fraction was evaluated.

MATERIALS AND METHODS

Venom and chemicals

The crude venom of E. carinatus was obtained from the Venomous Animals &from department of microbiology Hazara University, Mansehra (Pakistan). Sephadex G-75 and DEAE-Sepharose columns were purchased from Pharmacia (Sweden). CaCl2 and PT kit was purchased from Fisher Diagnostics (USA). Protein markers were obtained from BioRad (Hercules, USA). Other reagents and chemicals were of analytical grade from Fluka and Merck.

Blood collection

Normal plasma from 20 healthy donors without history of bleeding or thrombosis was collected from a private clinical laboratory. The citrated blood was centrifuged for 15 min at 3 000 rpm, to get clear plasma. The protrombin time (PT) was estimated.

Protein determination

Protein concentration was measured by the method of Lowry et al. (1951)[6], using BSA as standard.

Purification and isolation of anticoagulant factors

Lyophilized crude venom of E. carinatus (50 mg) was dissolved in 4 ml of ammonium acetate (20 mM ammonium acetate, pH 6.8) and centrifuged at 14 000 rpm for 15 min at 4˚C. Afterwards, the supernatant was filtered on a 0.45 µ filter to remove insoluble materials. The solution was applied to a 3 × 150 cm column packed with Sephadex G-75. The column was equilibrated with ammonium acetate buffer (pH 6.8) and then eluted with the same buffer. Fractions of 9 ml were collected at a flowfor clotting activity using human plasma[7,8].

Antigoagulant fractions were pooled and dialyzed overnight at 4˚C against 50 mM Tris/HCl buffer (pH 8.2) and applied on DEAE-Sepharose column (1.5 x 25 cm) equilibrated with 50 mM Tris/HCl, pH 8.2 and eluted with a linear gradient of NaCl concentration from 0.0 to 0.5 mM. Fractions of 6 ml were collected at 4˚C and a flow rate of 20 mL/h. The peaks were monitored at 280 nm[7,9].

Determination of molecular weight

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/ PAGE) was performed by the method of Laemmli (1970)[10].

Prothrombin time (PT) assay

For the PT test, 200 µL of the PT reagent was added to 100 µL of citrated plasma (incubated for 1 min at 37˚C). The time from the plasma-reagent mixing to clot formation was defined as the PT[11]. The PT test was performed for different concentration of crude venom and its fractions.

Anticoagulant activity of fractions

Normal plasma comprised mixed samples from 20 healthy donors. It was briefly incubated at 37˚C and sample aliquots containing the same concentration of anticoagulant fraction or subfraction (50 µg/ml) were added, mixed and shaken and then PT was recorded[11].

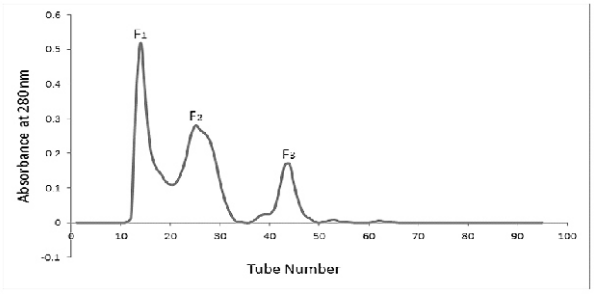

Figure-1. Sephadex G-75 chromatography of Echis carinatus venom

Concentrate of venom (mg/ml)	Average of PT (S)*	
Control	13.4 (P <0.005)	Clot complete
0.01	21 (P <0.001)	Clot is tiny
0.1	12.25(P <0.005)	Increased clot size
1	8.6 (P <0.001)	Clot complete

Table-I. PT value for different concentrations of E. carinatus crude venom

**n = 8. Total protein of the venom = 48300 µg/ml. Control = 100 µL of citrated plasma + 200 µL of the PT reagent + Normal saline (Instead of venom). Test = 100 µL of citrated plasma + 200 µL of the PT reagent + sample of venom.*

PT*	Fraction
12.3 s (P<0.05)	F1
35.5 s (P<0.01)	F2
More than 300 s	F3

Table-II. PT value for fractions of IEc crude venom

RESULTS

In order to study the anticoagulant activity of E. cari¬natus snake venom, the prothrombin time was estimated for different concentrations of crude venom. Our results indicated that plasma treated with crude venom coagulated very rapidly (Table-I), but after fractionation of the venom we noticed that some fractions could prolong the prothrombin time (Table-II).

Purification and isolation of anticoagulant factors

The crude venom was fractionated by gel filtration and three peaks (F1 to F3) were obtained (Fig. 1). Only fraction F2 showed anticoagulant activity (Table-II). Further purification was carried out by ion exchange chromatography on DEAE-Sepharose and those six frac¬tions were isolated. These fractions were labeled from F2A to F2F (Fig. 2). Out of six fractions only F2C and F2D fractions showed anticoagulant activity (Table-III). The anticoagulant fractions (F2C and F2D) isolated in the present work were characterized as proteases,

	Fraction
More than 300 s	Fraction F 2A
More than 300 s	Fraction F 2B
170 s (P < 0.05)	Fraction F 2C
280 s (P < 0.01)	Fraction F 2D
More than 300 s	Fraction F 2E
More than 300 s	Fraction F 2F

Table-III. PT value for F2 sub-fractions of E. carinatus venom

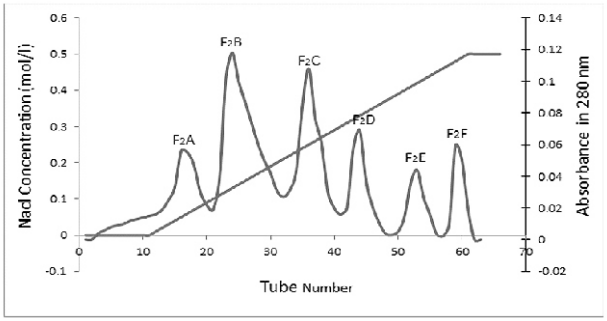

Figure-2. DEAE-Sepharose chromatography of F2 fraction obtained from Sephadex G-75.

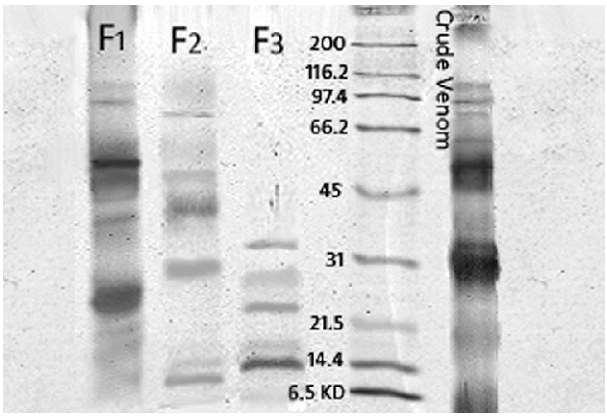

Figure 3. SDS/PAGE of crude venom and its fractions.
(A) Crude venom and its fractions

since a proteolytic effect was observed on casein, BAPNA or human plasma. Our results showed that the PT value significantly in¬creased in the F2C and F2D fractions as compared with PT value of the crude venom (Table- III).

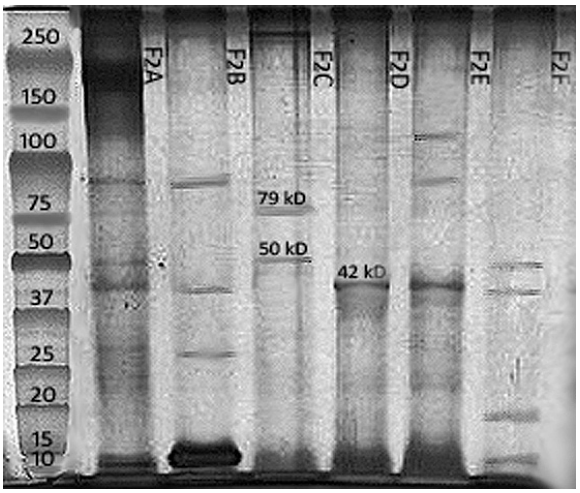

Figure 3. SDS/PAGE of crude venom and its fractions.

(B) Subfractions of F2.

Purity and determination of molecular weight

The crude venom and all fractions were analyzed by SDS/PAGE to estimate their protein composition (Fig. 3). The molecular weights of protein from the venom ranged from 6.5 to 250 kDa. The F2C fraction contained two protein bands of 50 and 79 kDa and F2D a single band a with molecular weight 42 kDa

DISCUSSION

The crude venom of the snake E. carinatus was assayed with PT test. The venom coagulated human plasma very rapidly, but its fractions delayed clotting. Thus it can be concluded that the venom contains anticoagulant factor(s). Purification and fractionation of snake venom has been carried out by several chromatography methods[12,13]. Here, anticoagulant factors were purified by a combination of gel filtration on Sephadex G-75 (Fig. 1) and ion-exchange chromatography on DEAE-Sepharose (Fig. 2). The present study describes an efficient and relatively simple process for isolation of anticoagulant factors (F2C and F2D) from the venom of Iranian Echis carinatus.

Snake venoms have different types of anticoagulant proteins, some of which have enzymatic activity, represented by phospholipase A2, metalloproteinases like a-fibrinogenase, serine proteinases and L-Amino acid oxidase. But others (C-type lectin-related proteins and three-finger toxins) do not show any enzymatic activity.

Some snake venoms contain toxins that are direct or indirect anticoagulants, which inhibit the clotting process, thus increasing the risk of bleeding[1]. Recently, the amino acid sequence of the ß-subunit of echi-cetin (from the venom of Echis carinatus) has been reported and found to belong to the snake venom subclass of the C-type lectin protein family[14]. An acidic phospholipase A2 was purified from Agkistrodon halys pallas venom by a two-step procedure of gel filtration on Sephadex G-100 and ion exchange chromatography on DEAE Sephadex A-50[15]. Another phospholipase A2 from Bothrops leucurus venom was purified by a three-step procedure involving gel filtration Sephacryl S-200, ion exchange chromatography Q-Sepharose and reverse phase HPLC Vydac C4 column[16]. Snake venom toxins that prolong blood coagulation are proteins or glycoproteins with molecular masses ranging from 6 to 350 kDa[2]. In this article, the molecular weights of purified anticoagulant factors were approximately 42, 50 and 79 kDa. Thus, these anticoagulant factors belong to the intermediate-molecular-weight group.

Some anticoagulant factors, along with their molecular weights, reported in the literature are: echicetin, isolated from the venom of Echis carinatus (saw-scaled viper), is composed of a 16-kDa a-subunit and a 14-kDa ß-subunit[14]. Lamino acid oxidase from Agkistrodon blomhoffii ussurensis has a molecular weight of 108.8 kDa[17] and metalloproteinase from Philodryas patagoniensis 53 kDa[3].

Several snake venom proteins with no 'detectable' (known or tested) enzymatic activity inhibit blood coagulation. A number of non-enzymatic anticoagulant proteins have been purified and characterized. These proteins inhibit the coagulation process through their direct interaction with a specific coagulation factor. The mech¬anisms appear to be simple, and these

proteins interfere in either complex formation or inhibit the activity of one of the proteinases[2]. The study of such factors significantly contributes to our understanding of blood coagulation. Furthermore, the structure–function relationships of these proteins and identification of the functional sites may be useful in the development of new anticoagulant agents.

It seems that the anticoagulant factors isolated from E. carinatus inhibit platelet aggregation in a manner similar to human von Willebrand factor (vWf) and echicetin.

CONCLUSIONS

In this study we demonstrated that IEc venom contains both coagulant and anticoagulant activities. The anticoagulant activity of these fractions (F2C and F2D) on human plasma could be caused by proteolytic enzymes. Further studies are needed to verify this hypothesis.

In the present study the venom of Echis carinatus was fractionated by chromatography and each fraction evaluated by PT test. These fractions showed enzymatic activity. Their main component were proteins of molecular weights of about 42, 50 and 79 kDa.

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"Initiative is doing the right thing without being told."

Victor Hugo