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Isolation and partial purification of anticoagulant fractions from the venom of the Iranian snake *Echis carinatus*

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Many snake venoms comprise different factors, which can either promote or inhibit the blood coagulation pathway. Coagulation disorders and hemorrhage belong to the most prominent features of bites of the many vipers. A number of these factors interact with components of the human blood coagulation. This study is focused on the effect of *Echis carinatus* snake venom on blood coagulation pathway. Anticoagulant factors were purified from the Iranian *Echis carinatus* venom by two steps: gel filtration (Sephadex G-75) and ion-exchange (DEAE-Sephadex) chromatography, in order to study the anticoagulant effect of crude venom and their fractions. The prothrombin time was estimated on human plasma for each fraction. Our results showed that protrombin time value was increase from 13.4 s to 170 s for F2C and to 280 s for F2D. Our study showed that these fractions of the venom delay the prothrombine time and thus can be considered as anticoagulant factors. They were shown to exhibit proteolytic activity. The molecular weights of these anticoagulants (F2C, F2D) were estimated by SDS/PAGE electrophoresis. F2C comprises two protein bands with molecular weights of 50 and 79 kDa and F2D a single band with a molecular weight of 42 kDa.

Key words: snake venom, Iranian *Echis carinatus*, anticoagulant factor, chromatography

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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 (Julian, 2005).

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 (Kini, 2006).

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 (Kini, 2005; 2006; Morita, 2005).

In the present study the venom of Iranian 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 Iranian *E. carinatus* was obtained from the Department of Venomous Animals and Antivenom Production, Razi Vaccine and Serum Research Institute Karaj, Iran. Sephadex G-75 and DEAE-Sepharose columns were purchased from Pharmacia (Sweden). $CaCl_2$ 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 3000 rpm, to get clear plasma. The protrombin time (PT) was estimated (Ghorbanpur *et al.*, 2010).

Protein determination. Protein concentration was measured by the method of Lowry *et al.* (1951), 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 14000 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 flow

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Abbreviations: IEc, Iranian snake *Echis carinatus;* vWf, human von Willebrand factor

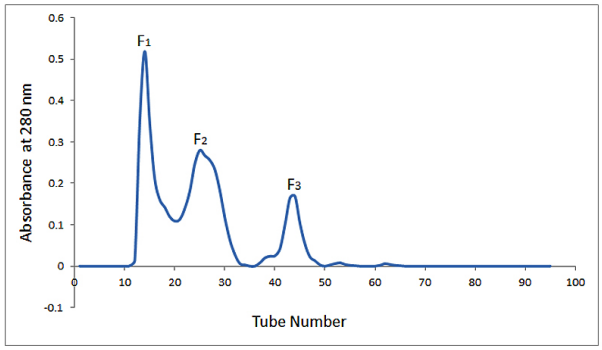

Figure 1. Sephadex G-75 chromatography of *Echis carinatus* venom.

rate of 60 mL/hour at 4°C. Fractions were analyzed for clotting activity using human plasma (Daisuke *at al.*, 1996; Loubna *at al.*, 2010).

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 (Daisuke *at al.*, 1996; Qing-qiu *at al.*, 1999).

Determination of molecular weight. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) was performed by the method of Laemmli (1970).

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 (Ghorbanpur *et al.*, 2010; Rizzo *at al.*, 2008). 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 (Ghorbanpur *et al.*, 2010; Rizzo *at al.*, 2008).

RESULTS

In order to study the anticoagulant activity of *E. carinatus* 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 1), but after fractionation of the venom we noticed that some fractions could prolong the prothrombin time (Table 2).

Table 1. PT value for different concentrations of *E. carinatus* crude 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

*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.

Table 2. PT value for fractions of *IEc* crude venom

Fraction	PT*
F_1	12.3 s ($P<0.05$)
F_2	35.5 s ($P<0.01$)
F_3	More than 300 s

*n=4, normal PT=13.4 s. Total protein of F_2=276 µg/ml

Table 3. PT value for F_2 sub-fractions of *E. carinatus* venom

Fraction	PT *
Fraction F_2A	More than 300 s
Fraction F_2B	More than 300 s
Fraction F_2C	170 s ($P < 0.05$)
Fraction F_2D	280 s ($P < 0.01$)
Fraction F_2E	More than 300 s
Fraction F_2F	More than 300 s

*n=4. Total protein of F_2C=97 µg/ml and F_2D=64 µg/ml

Purification and isolation of anticoagulant factors

The crude venom was fractionated by gel filtration and three peaks (F_1 to F_3) were obtained (Fig. 1). Only fraction F_2 showed anticoagulant activity (Table 2).

Further purification was carried out by ion exchange chromatography on DEAE-Sepharose and those six fractions were isolated. These fractions were labeled from F_2A to F_2F (Fig. 2). Out of six fractions only F_2C and F_2D fractions showed anticoagulant activity (Table 3).

The anticoagulant fractions (F_2C and F_2D) isolated in the present work were characterized as proteases, since a proteolytic effect was observed on casein, BAPNA or human plasma.

Our results showed that the PT value significantly increased in the F_2C and F_2D fractions as compared with PT value of the crude venom (Table 3).

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 F_2C fraction contained two protein bands of 50 and 79 kDa and F_2D a single band a with molecular weight 42 kDa.

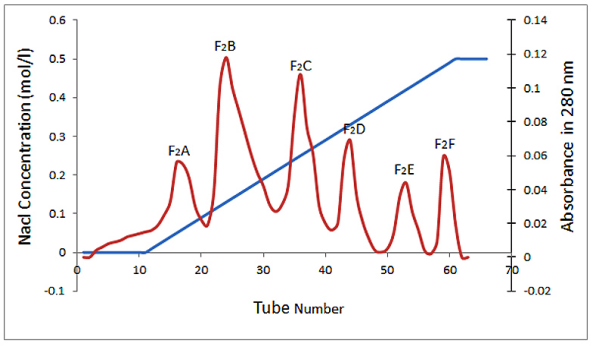

Figure 2. DEAE-Sepharose chromatography of F_2 fraction obtained from Sephadex G-75.

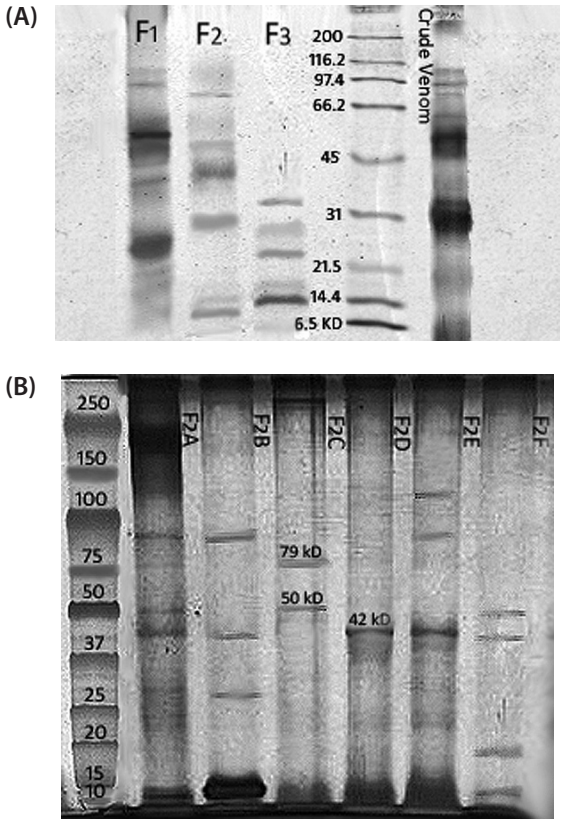

Figure 3. SDS/PAGE of crude venom and its fractions.
(A) Crude venom and its fractions, **(B)** Subfractions of F_2.

DISCUSSION

The crude venom of the Iranian 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 (Ghorbanpur *et al.*, 2010; Peichoto *at al.*, 2007; Oyama *at al.*, 2003). 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 (F_2C and F_2D) from the venom of Iranian *Echis carinatus*.

Snake venoms have different types of anticoagulant proteins, some of which have enzymatic activity, represented by phospholipase A_2, metalloproteinases like α-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 (Ghorbanpur *et al.*, 2010).

Some snake venoms contain toxins that are direct or indirect anticoagulants, which inhibit the clotting process, thus increasing the risk of bleeding (Julian W, 2005). Recently, the amino acid sequence of the β-subunit of echicetin (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 (Janos *at al.*, 1997). An acidic phospholipase A_2 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 (Wang *at al.*, 2007). Another phospholipase A_2 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 (Higuchi *at al.*, 2007).

Snake venom toxins that prolong blood coagulation are proteins or glycoproteins with molecular masses ranging from 6 to 350 kDa (Kini, 2006). 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 α-subunit and a 14-kDa β-subunit (Janos *at al.*, 1997). L-amino acid oxidase from *Agkistrodon blomhoffii ussurensis* has a molecular weight of 108.8 kDa (Wei *at al.*, 2007) and metalloproteinase from *Philodryas patagoniensis* 53 kDa (Kini, 2005).

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 mechanisms appear to be simple, and these proteins interfere in either complex formation or inhibit the activity of one of the proteinases (Kini, 2006). 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.

In this study we demonstrated that IEc venom contains both coagulant and anticoagulant activities. The anticoagulant activity of these fractions (F_2C and F_2D) on human plasma could be caused by proteolytic enzymes. Further studies are needed to verify this hypothesis.

CONCLUSIONS

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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