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Blood Coagulation Induced by Iranian Saw-Scaled Viper (*Echis Carinatus*) Venom: Identification, Purification and Characterization of a Prothrombin Activator

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ABSTRACT

Objective(s): *Echis carinatus* is one of the venomous snakes in Iran. The venom of Iranian *Echis carinatus* is a rich source of protein with various factors affecting the plasma protein and blood coagulation factor. Some of these proteins exhibit types of enzymatic activities. However, other items are proteins with no enzymatic activity.

Materials and Methods: In order to study the mechanism and effect of the venom on human plasma proteins, the present study has evaluated the effect of crude venom and all fractions. A procoagulant factor (prothrombin activator) was isolated from the venom of the Iranian snake *Echis carinatus* with a combination of gel filtration (Sephadex G-75), ion-exchange chromatography (DEAE- Sepharose) and reverse phase HPLC. Furthermore, proteolytic activity of the crude venom and all fractions on blood coagulation factors such as prothrombin time (PT) was studied.

Results: In the present study, the PT test was reduced from 13.4 s to 8.6 s when human plasma was treated with crude venom (concentraion of venom was 1 mg/ml). The purified procoagulant factor revealed a single protein band in SDS polyacrylamide electrophoresis under reducing conditions and its molecular weight was estimated at about 65 kDa. A single-band protein showed fragment patterns similar to those generated by the group A prothrombin activators, which convert prothrombin into meizothrombin independent of the prothrombinase complex.

Conclusion: This study showed that the fraction which separated from Iranian snake *Echis carinatus* venom can be a prothrombin activators. It can be concluded that this fraction is a procoagulant factor.

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Introduction

Snake venom, a complex mixture principally composed of proteins and peptides, exhibits diverse biological activities that affect several vital systems (1).

Echis carinatus (Saw scaled viper) is a venomous snake found in the desert regions of Iran. The venom of *E. carinatus*, a member of the Viperidae family, affects blood coagulation due to hemostatically active enzymes with procoagulant and anticoagulant activity (2, 3).

The venom of *E. carinatus* affects the blood circulation. This venom is very toxic causing severe tissue and organ damage. The venom of *E. carinatus* is rich in proteins and peptides effective on the hemostatic system, i.e., its acts against some types of factors involving coagulation and fibrinolysis (4, 5).

E. carinatus snake venom especially contains proteins affecting the transformation of the prothrombin into thrombin (6). Prothrombin is the protein which is broken in plasma by ecarin. In fact, this protein cleaves the bond in prothrombin and produces meizothrombin, which is converted into α-thrombin by autolysis (7).

The conversion of the prothrombin into thrombin is one of the central reactions of blood coagulation (8, 9). The physiological activation of prothrombin to the serine proteinase α-thrombin is catalysed by prothrombinase complex consisting of the serine proteinase, factor Xa, cofactor Va and Ca^{2+}. Membranes containing anionic phospholipids are essential for the optimal function of this enzyme complex (10, 11). However, the rate of activation is five orders of magnitude lower than the activation by prothrombinase complex (12), and the mechanism of cleavage proceeds through prethrombin-2 rather than through meizothrombin (13).

The venom of Viperidae presents a high level of

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haemorrhagic, coagulant and proteolytic activities (14). Proteins effective on blood coagulation and existing in the snake venom are classified based on their ability to lengthen or shorten the clotting process into coagulation and anticoagulation proteins (15).

The aim of the present investigation was to study the purification and characterization of porothrombin activator (procoagulant factor) from the Iranian *E. carinatus* venom and to evaluate the procoagulant activity on *in vitro* human plasma.

Materials and Methods

Material

The lyophilized *E. carinatus* venom was obtained from the Department of Venomous Animals and Antivenom Production, Razi Vaccine and Serum Research Institute Karaj, Iran. Sephadex G-75, DEAE-Sepharose, and C18 columns were purchased from the Pharmacia company (Sweden). $CaCl_2$ and PT kits were purchased from the Fisher Diagnostics (USA). Protein markers were obtained from BioRad (Hercules, USA). Other reagents and chemicals were of analytical grade from Fluka and Merck.

Methods

Blood collection

Normal plasma from 20 healthy donors without any 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. Finally, the PT was estimated.

Protein determination

The total protein of crude venom of *E. carinatus*, and its fractions were determined by Lowry method (16).

Purification and isolation of prothrombin activator

Purification of the prothrombin activator was performed in three steps. Lyophilized crude venom of *E. carinatus* (50 mg) was dissolved in 4 ml of starting buffer (20 mM ammonium acetate, pH 6.8) and centrifuged at 3,000 rpm for 15 min, 4°C. The supernatant was filtered on a 0.45 microfilter to remove all insoluble materials. The supernatant was then applied into a Superdex G-75 column and eluted with the same buffer. (150 × 3 cm). Fractions were collected at 4°C and their absorbances were recorded at 280 nm. The fractions with proguaolant activity were pooled, lyophilized and dialyzed against 50 mM Tris-HCl, pH 8.2 buffers. The dialyzed sample was centrifuged at 3000 rpm to clear the precipitated proteins. For further purification, the supernatant was loaded into ion exchange column (DEAE-Sepharose) and equilibrated with 50 mM Tris-HCl buffer, pH 8.2 and eluted with a liner gradient of Nacl concentration from 0.0 to 0.5 mM. The fractions exhibiting proguaolant activity in the previous step were pooled and dialyzed overnight at 4°C and applied on HPLC column, C18 (H_2O, 0.1% trifluoroacetic acid), and eluted with a concentration gradient of solvent B (acetonitrile, 0.1% trifluoroacetic acid) from 0 to 100%, at a flow rate of 0.3 ml/min during 55 min. The peaks were monitored at 280 nm (17).

Determination of molecular weights

Electrophoresis on 12/5% polyacrylamide gel was performed according to the method of Laemmli (18). Samples of the crude venom and its fractions were lauded and the molecular weights of protein were determined under reduced conditions.

Prothrombin time 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 the clot formation was defined as the PT and clotting time was recorded (19). The PT test was performed for different concentrations of crude venom and its fractions.

Coagulant activity

Normal plasma comprised mixed samples from 20 healthy donors. It was briefly incubated at 37°C and sample aliquots containing some concentration of coagulant fractions or subfraction (50 µg/ml) were added, mixed and shaken and PT was then recorded.

Results

The present study showed that the crude venom of *E. carinatus* can accelerate the blood coagulation pathway. Our results indicated that as the concentration of venom increases, the PT of plasma decreased (Table 1).

According to the Table 1, when the concentrations of venom increased from 0.01 to 1 mg/ml, the clotting time of plasma reduced from 13.4 to 8.6 sec.

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

Concentrate of venom (mg/ml)	Average of PT (S) *	Preamble
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
Control	13.4 ($P < 0.005$)	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 + different concentrate of venom.

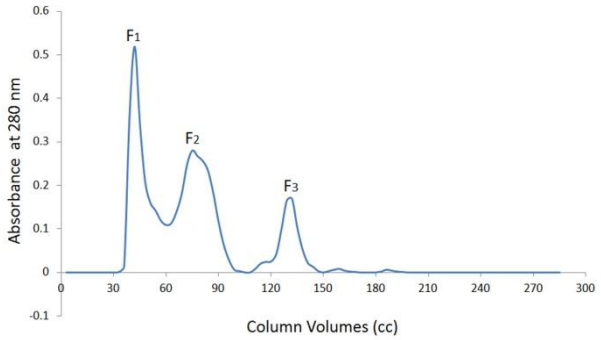

Figure 1. Purification of crude venom of *Echis carinatus* by Sephadex G-75

Table 2. PT value for fractions of I*Ec* crude venom

Fractions	PT *
F_1	12.3 sec
F_2	35.5 sec
F_3	More than 300 sec

* n=4, normal PT=13.4, F_1 ($P < 0.05$) and F_2 ($P < 0.01$)
PT: prothrombin time

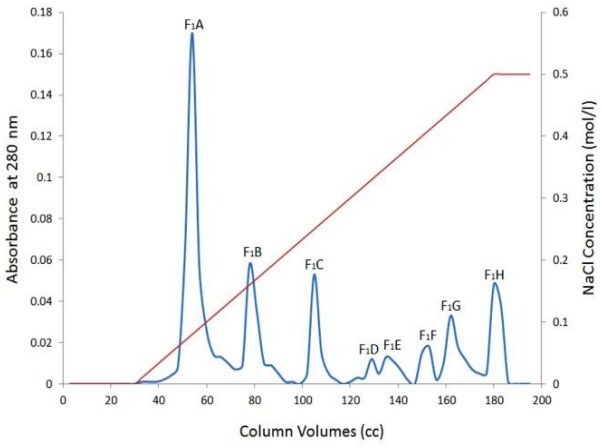

Figure 2. Purification of F_1 by DEAE-Sepharose chromatography

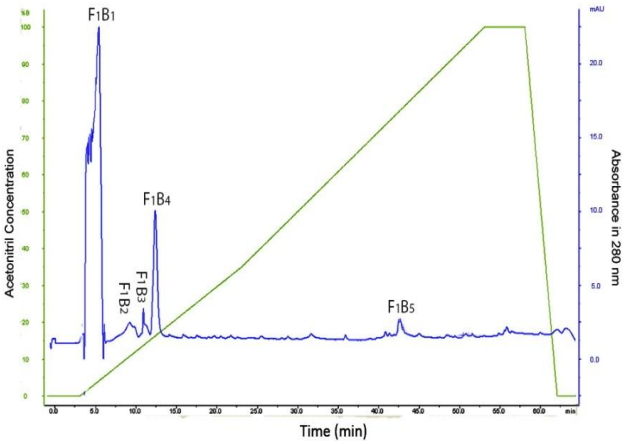

Figure 3. HPLC of F_1B fraction obtained from DEAE-Sepharose chromatography

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

Fractions	PT *
Fraction F_1A	14 sec
Fraction F_1B	8 sec
Fraction F_1C	70 sec
Fraction F_1D	52 sec
Fraction F_1E	90 sec
Fraction F_1F	56 sec
Fraction F_1G	More than 300 sec
Fraction F_1H	95 sec

* n=4, F_1B (P-Value < 0.05)
PT: prothrombin time

Table 4. Prothrombin Time for fractions obtained from HPLC

Fractions	Average of PT *
Fraction F_1B_1	More than 300 sec
Fraction F_1B_2	More than 300 sec
Fraction F_1B_3	More than 300 sec
Fraction F_1B_4	3 sec
Fraction F_1B_5	More than 300 sec

* n=4, F_1B_4 (P-Value < 0.05)
PT: prothrombin time

Table 5. Summerized PT value and total protein (crude venom, F_1, F_1B, F_1B_4)

Step	Protein
Venom	48.3 mg/ml
F_1	387.77 µg/ml
F_1B	130 µg/ml
F_1B_4	26 µg/ml

PT: prothrombin time

According to the Table 1, when the concentrations of venom increased from 0.01 to 1 mg/ml, the clotting time of plasma reduced from 13.4 to 8.6 sec.

Purification, isolation and characterization of prothrombin activator

As it is shown in the Figure 1, the three fractions (F_1 to F_3) were obtained By Sephadex G-75. Prothrombin time value was estimated for all the fractions. Our observation showed that the PT value for F_1 is less than other fractions and this fraction can be considered as a procoagulant factor (Table 2).

Further purification was performed by ion exchange chromatography DEAE-Sepharose. In this step, eight fractions were separated from F_1A to F_1H (Figure 2), out of eight fractions, only F_1B showed procoagulant activity (Table 3).

The F_1B was pooled, dialyzed and applied to a C18 reversed-phase HPLC column. Our results revealed that five peaks from F_1B_1 to F_1B_5 were isolated (Figure 3) and out of five fractions, only F_1B_4 showed coagulant activity (Table 4).

Our results summarized in the Table 5, which showed that the PT value significantly decreased in the F_1B_4 as compared with PT value of the crude venom.

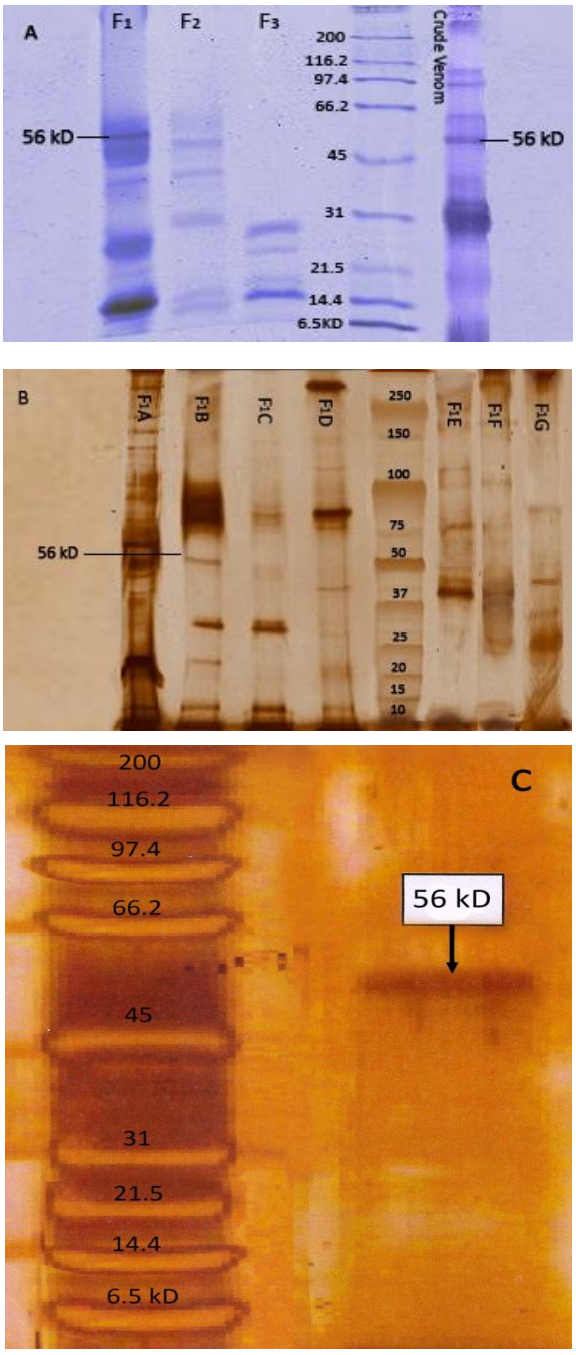

Figure 4. SDS-PAGE pattern of crude venom and its fraction: **A:** Crude venom and its fractions; **B:** Subfractions of F_1; **C:** Fraction of F_1B_4

Purity and determination of molecular weight

Crude venom and all fractions were analyzed by SDS-PAGE. As it is shown in the SDS-PAGE pattern, the molecular weight of crude venom and all of the fractions were estimated (Figure 4A, 4B and 4C). The molecular weights from the snake venom ranged from 6.5 to 250 kDa and the molecular weight of procoagulant factor was approximately 56 kDa. According to the Figure 4C, a single band of F_1B_4 indicates the purity of this protein.

Discussion

This study has investigated the venom of Iranian *E. carinatus* which contains a strong procoagulant factor enabling to activate the prothrombin. The functional properties of the *E. carinatus* prothrombin activator are similar to ecarin, the first prothrombin activator which was recently discovered to be present in the venom from *E. carinatus* (20). The venoms of many *E.* species are able to convert prothrombin into thrombin, either directly or indirectly (21).

Under *in vitro* conditions, this venom also displays coagulation properties and increases the blood coagulation cascade. Crude venom from the Iranian snake *E. carinatus* was selected and assayed with PT test. Our results indicated that the Iranian *E. carinatus* venom has a procogulant activity and is able to coagulate human plasma rapidly (Table 1), therefore it may be concluded that I*Ec* venom contains procoagulant factors.

The present study reports an efficient and simple procedure for purification and isolation of procoagulant factor from I*Ec* venom. The fraction F_1B_4 was isolated from I*EC* venom by a combination of several methods. Our results revealed that three peaks from F_1B_1 to F_1B_5 were isolated. In addition, out of five fractions, only F_1B_4 showed coagulant activity (Table 4). The molecular weight of this purified fraction was approximately estimated to be 56 kDa (Figure 4C). Our observation showed that the molecular weight of F_1B_4 is similar to prothrombin activator enzymes which have been already reported (20). Therefore, this coagulant factor may belong to the intermediate-molecular-weight group of these factors.

By performing the prothrombin Time test on human plasma, the blood coagulation time on fraction F_1 showed the least coagulation time and fraction F_3 displayed the highest coagulation time. The total protein of crude venom is 48.3 mg/ml and the PT value is 8.6 s but in the hyper purified fraction with reducing amount of total protein (26.0 µg/ml), the PT value (3 s) also significantly decreased. It may be suggested that with low amount of total protein the PT value decreases.

Some procoagulant factors, along with its molecular weights, have been reported by Howes JM *et al* in addition to the effects of three novel metallo-proteinases (weighting 56 kDa) from the venom of the West African saw-scaled viper, *E. ocellatus* on blood coagulation and platelets (22). Daisuke Yamada *et al* isolated and characterized the carinactivase, a novel prothrombin activator from *E. carinatus* Venom with 62 kDa (23).

Mikarin is the first group of IA prothrombin activator identified in the venom of a viperidae snake. In the case of prothrombin activator, it exhibited prothrombin activation, which was similar

to the other group IA prothrombin activators, such as ecarin from *E. Carinatus* (24), aharin from *Agkistrodon halys pallas* (25) and prothrombin activator from *Bothrops atrox* (26).

Over the past 20 years, many metalloproteinase have been isolated from snake venom with a wide variety of biological activities, including hemorrhagic (27), fibrinogenolytic and antiplatelet effects (28), as well as activation of prothrombin and factor X (29).

Conclusion

Protein with coagulation activities was purified from the venom of *E. carinatus*. The venom of *E. carinatus* including the Iranian *E. carinatus* is one of the coagulation venoms whose function is a pseudothromboplastin action. However, under *in vitro* conditions, this venom will generate high coagulation which is due to activation of the prothrombin.

It is suggested that, this venom containing procoagulant factors with molecular weight of about 56 kDa. It seems the fraction F_1B_4 isolated from I*Ec* to be like ecarin which is already reported.

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