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

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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 (concentration 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 activator. It can be concluded that this fraction is a procoagulant factor.

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 Ca²⁺. 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 prothrombin-2 rather than through meizothrombin (13).

The venom of Viperidae presents a high level of
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 prothrombin 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). CaCl2 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 procoagulant 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 linear gradient of NaCl concentration from 0.0 to 0.5 mM. The fractions exhibiting procoagulant activity in the previous step were pooled and dialyzed overnight at 4°C and applied on HPLC column, C18 (H2O, 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 = 483000 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.
Table 2. PT value for fractions of E. carinatus crude venom

| Fractions | PT * |
|-----------|------|
| F₁        | 12.3 sec |
| F₂        | 35.5 sec |
| F₃        | More than 300 sec |

* n=4, normal PT=13.4, F₁ (P < 0.05) and F₂ (P < 0.01)

PT: prothrombin time

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

| Fractions | PT * |
|-----------|------|
| Fraction F₁A | 14 sec |
| Fraction F₁B | 8 sec |
| Fraction F₁C | 70 sec |
| Fraction F₁D | 52 sec |
| Fraction F₁E | 90 sec |
| Fraction F₁F | 56 sec |
| Fraction F₁G | More than 300 sec |
| Fraction F₁H | 95 sec |

* n=4, F₁B (P-Value < 0.05)

PT: prothrombin time

Table 4. Prothrombin Time for fractions obtained from HPLC

| Fractions | Average of PT * |
|-----------|-----------------|
| Fraction F₁B₁ | More than 300 sec |
| Fraction F₁B₂ | More than 300 sec |
| Fraction F₁B₃ | More than 300 sec |
| Fraction F₁B₄ | 3 sec |
| Fraction F₁B₅ | More than 300 sec |

* n=4, F₁B₄ (P-Value < 0.05)

PT: prothrombin time

Table 5. Summerized PT value and total protein (crude venom, F₁, F₁B, F₁B₄)

| Step | Protein |
|------|---------|
| Venom | 48.3 mg/ml |
| F₁   | 387.77 µg/ml |
| F₁B  | 130 µg/ml |
| F₁B₄ | 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 (F₁ to F₃) were obtained by Sephadex G-75. Prothrombin time value was estimated for all the fractions. Our observation showed that the PT value for F₁ 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 F₁A to F₁H (Figure 2), out of eight fractions, only F₁B showed procoagulant activity (Table 3).

The F₁B was pooled, dialyzed and applied to a C18 reversed-phase HPLC column. Our results revealed that five peaks from F₁B₁ to F₁B₅ were isolated (Figure 3) and out of five fractions, only F₁B₄ showed coagulant activity (Table 4).

Our results summarized in the Table 5, which showed that the PT value significantly decreased in the F₁B₄ as compared with PT value of the crude venom.
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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 procoagulant activity and is able to coagulate human plasma rapidly (Table 1), therefore it may be concluded that IEc venom contains procoagulant factors.

The present study reports an efficient and simple procedure for purification and isolation of procoagulant factor from IEc venom. The fraction F1B4 was isolated from IEC venom by a combination of several methods. Our results revealed that three peaks from F1B1 to F1B5 were isolated. In addition, out of five fractions, only F1B4 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 F1B4 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 F1 showed the least coagulation time and fraction F3 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.

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 F1B4 indicates the purity of this protein.

Figure 4. SDS-PAGE pattern of crude venom and its fraction: A: Crude venom and its fractions; B: Subfractions of F1; C: Fraction of F1B4.
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 F1:B1 isolated from 1Ec to be like ecarin which is already reported.

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