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CHARACTERIZATION OF AN ANTICOAGULANT AGENT IN THE VENOM OF IRANIAN COBRA (NAJA NAJA OXIANA).

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ABSTRACT

Snake venoms contain a rich variety of factors which affect blood coagulation. There have been few reports on the anticoagulant activity of the venom of different cobras, but no such observations have been made on the Iranian cobra, although a procoagulant factor has been described.

In this study an anticoagulant factor has been purified from the venom of *Naja naja oxiana* using gel filtration followed by isopycnic ultracentrifugation with 30% KBr. The factor was a glycoprotein with a molecular weight of 45.36 kDa and increased plasma recalcification time by 224 sec which was 7.7 times greater than the activity of crude venom.

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INTRODUCTION

Snake venom contains a rich variety of factors affecting hemostatic mechanisms and can be classified broadly as possessing coagulant, anticoagulant and hemorrhagic activity.[1,2] For these reasons, snake venoms and their pure enzymes are being used in clinical diagnosis of abnormalities of blood clotting or as drugs in disturbances of hemostasis.[3-5] For example, Russell viper venom is used as an activator of factor X and used for the diagnosis of Stuart Perus' disease (lack of factor X in blood clotting). The ability of a strong procoagulant fraction in *E. carinatus* venom to convert prothrombin to thrombin in the absence of calcium ion has been used for development of a sensitive clotting assay to measure the prothrombin level.[6,7] It has also been seen that a fibrinolytic agent is present in the venom. *In vivo* studies have shown that injection of purified prothrombin activator from *E. carinatus* venom ecarin prevents arterial

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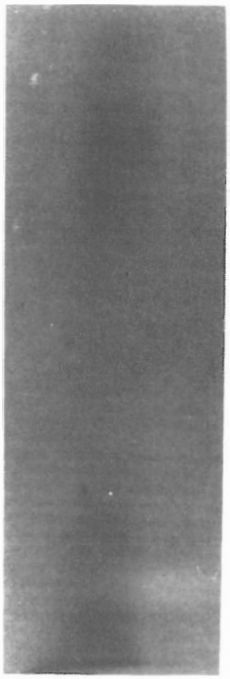


Fig. 1. SDS-PAGE of crude venom in 7.5% gel stained with Coomassie blue R-250.

or venous thrombus formation in animals given electric shock or kaolin, respectively for production of experimental thrombosis. This could be due to rapid depletion of fibrinogen caused by the procoagulating action of ecarin or it might be due to the fibrinogenolytic activity of ecarin. It is suggested that ecarin might be useful in the prevention or treatment of thrombin disease.[8-10]

No report has been made on any anticoagulant component in the venom of the Iranian cobra *Naja naja oxiana*, although an activator of factor V which induced clotting has been detected.[11] It was decided to purify an anticlotting factor from the venom of *Naja naja oxiana*.

MATERIALS AND METHODS

Lyophilized venom from *Naja naja oxiana* was obtained from Razi Institute, Tehran. Care was taken to see that the venom was always from the first milking. The venom was disolved in PBS and protein estimation done by Lowry's method.[12]

Gel filtration chromatography

The venom was chromatographed on Sephadex G-200 (1000 cm × 3.0 cm, void volume 235.5 mL) using 1M Tris-HCl, pH 8.0 with 0.001% sodium azide as preservative. 10mL buffer containing 250 mg venom after centrifugation at 5,000 RPM for 10 min was loaded and 75 × 5 mL fractions were collected at a flow rate of 16 mL/h and their absorbance recorded at 280 nm using a Shimadzu spectrophotometer.

All the stages were carried out at 10°C. The fractions were tested for anticlotting activity by plasma recalcification time. Fractions which were positive for anticlotting activity were lyophilized and different concentrations checked for anticlotting activity and were further purified by isopycnic ultracentrifugation.

Isopycnic ultracentrifugation

Isopycnic ultracentrifugation was carried out using 30% KBr at 110,000 Amax for 20h in a Beckman centrifuge XL100. 10 × 10 mL fractions were collected and their absorbance read at 280 nm. Fractions were dialyzed against PBS pH 7.2 concentrated by dialysis in a solution of 10% PEG 6000 and protein estimated by Lowry's method and tested for anticlotting activity once more.

Plasma recalcification time

Recalcification times were measured at 37°C on human

Table I. Plasma recalcification time for whole venom.

Protein (µg/mL)	Clotting time for control	Clotting time for venom	Delay in clotting
100	2' 27"	2' 58"	29"
50	2' 26"	2' 26"	19"

Table II. Anticlotting activity with different concentrations of fractions obtained from gel filtration.

Concentration (µg/mL)	Delay in blood clotting in seconds			
	F1	F2	F3	F4
500	-45	25	56	26
200	-24	9	32	21
100	-13	3	13	7
50	-9	0	8	5

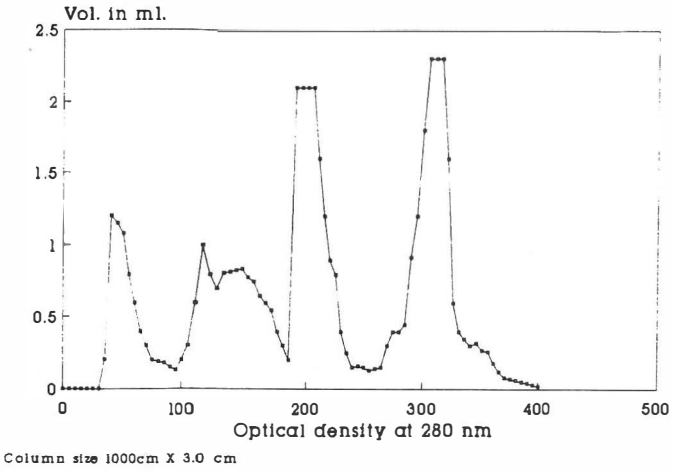


Fig. 2. Gel filtration of *Naja naja oxiana* venom with Sephadex G-100.

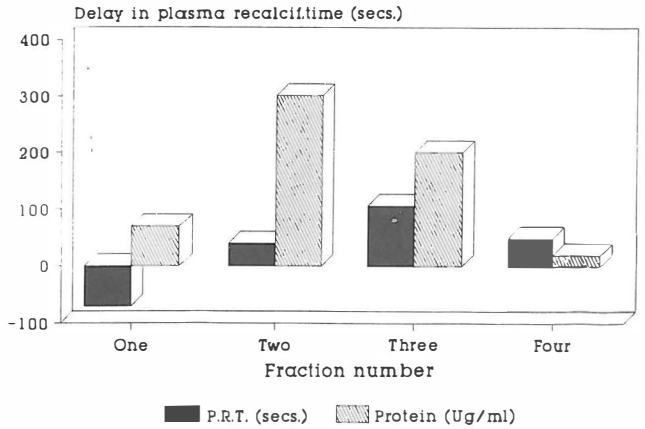
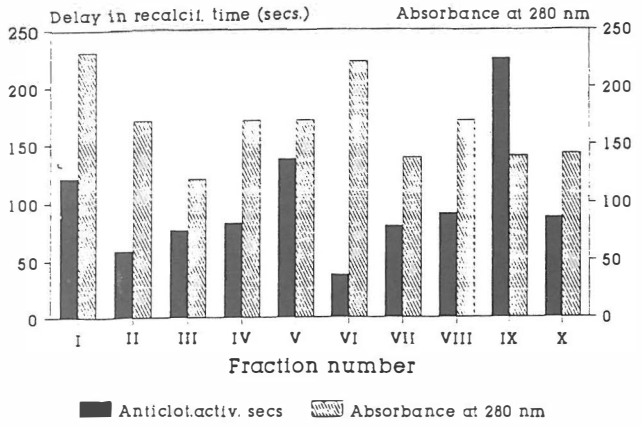


Fig. 3. Anticlotting activity and protein of peaks obtained from gel filtration.



Each frs is 10 ml from low-high density

Fig. 4. Anticlotting activity and absorbance of fractions from isopycnic ultracentrifugation.

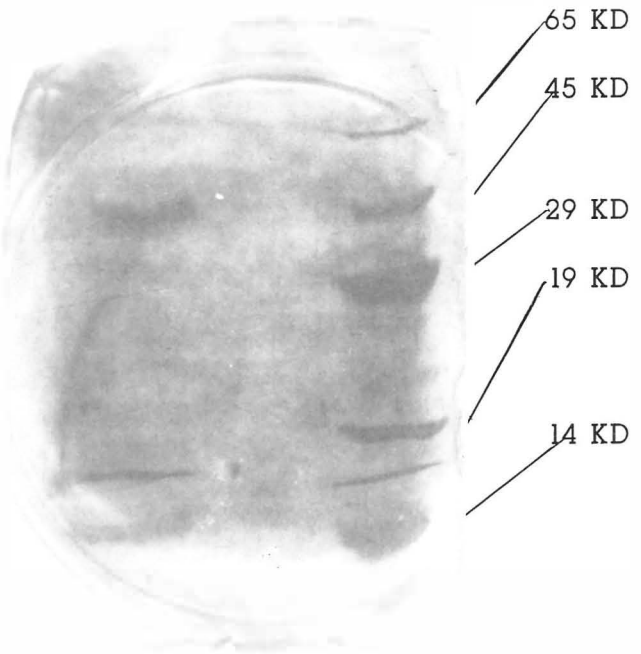


Fig. 5. SDS-PAGE of pure anticlotting factor on 10% gel stained with silver.

platelet poor plasma obtained from human citrated (1: 9 v/v) blood.[19] Veronal buffer containing 0.026 M sodium barbital/sodium acetate/0.1 M sodium chloride, pH 7.3 was used for diluting the venom and also as control. Fresh controls were used for each test.[19]

SDS-Polyacrylamide gel electrophoresis

This was carried out by the method of Laemmeli.[14] The venom in different stages of purification was electrophoresed on 10% gel under reducing conditions with standard molecular weights of bovine serum albumin 65 kDa, ovalbumin 45 kDa, carbonic anhydrase 29 kDa,

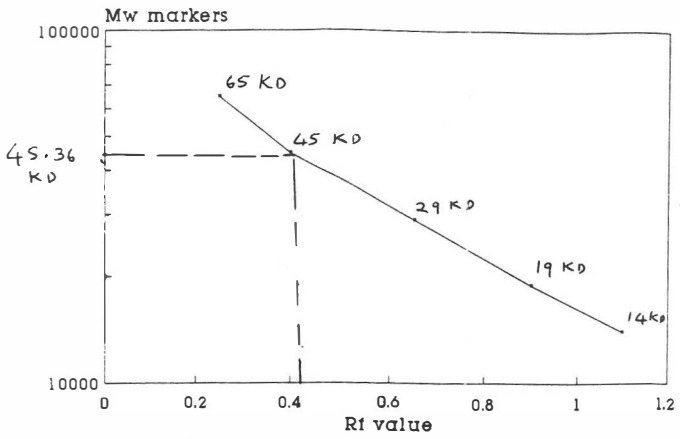


Fig. 6. Rf values of standard molecular weight markers.

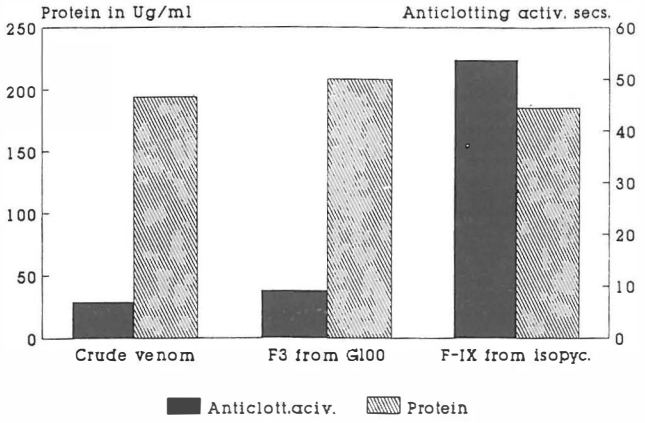


Fig. 7. Comparison of anticlotting activity of different stages of purification of agents from venom of *Naja naja oxiana*.

lactoglobulin 19 kDa, and lactalbumin 14 kDa. The gel was then stained with Coomassie blue R-250 or with silver by the method of Blum et al.[15] depending on the protein loaded. Similar gels were checked for polysaccharide with Schiff's staining[16] and for lipids with Sudan Black.

RESULTS

97% of lyophilized venom was protein. A plasma recalcification test was carried out on the whole venom and it postponed clotting time by 30 seconds (Table I). SDS-PAGE of whole venom revealed more than 17 bands as seen by Coomassie blue staining (Fig. 1). On purification with Sephadex G-100, four peaks were obtained (Fig. 2) which were tested for anticlotting action and it was the third peak which exhibited the most anticlotting effect (Fig. 3 and Table II). SDS-PAGE of this peak revealed three bands by silver staining. This fraction was subjected to isopycnic ultracentrifugation and the ten fractions obtained were dialyzed to remove KBr, concentrated (Fig. 4) and tested for anticlotting activity. The fifth fraction in the middle of the tube showed the most anticlotting activity (Fig. 4).

SDS-PAGE of the same showed a single band at 45.36 kDa (Fig. 5) by silver staining[15] (Fig. 6). PAS staining indicated a glycoprotein. This pure agent increased plasma recalcification time by 224 seconds which was 7.7 times greater than the activity of crude venom by plasma recalcification test (Fig. 7).

DISCUSSION

The anticlotting factor purified from *Naja naja oxiana* has a molecular weight of 45.36 kDa and is a glycoprotein as shown by PAS staining and increased clotting time by 224 seconds by plasma recalcification time. Assays in use for anticlotting are bleeding time (B.T.), prothrombin time, partial thromboplastin time and recalcification time. From these tests, only PT, APTT and recalcification time increase when any of the stages of blood clotting are disturbed. APTT is normally less than 50 seconds whereas recalcification time of platelet poor plasma is between 90-250 seconds, hence recalcification time was used in our assay and it was seen that our anticlotting factor with 44.5 µg/mL of protein delayed clotting by 224 seconds. A similar test was used with whole venom of *Naja naja atra* which showed strong anticlotting activity. The pure anticlotting factor was seen to be a phospholipase A which delayed clotting by 514 seconds.[17] Phospholipase A_2 has been purified from the venom of other snakes and it exhibits anticoagulant activity besides other properties. Phospholipase A_2 has been identified in the venom of elapids like *Naja naja naja* and *Naja mossambica*,[18] but it has been observed that the molecular weight of these enzymes were less than 18 kDa. However, no anticlotting factor has been purified from *Naja naja oxiana* but an activator of bovine factor V was purified from it's venom.[11] We too have observed a similar activity from peak two of the Sephadex G 100 column which decreased clotting time by 106 secs as compared to normal.

The molecular weight of the anticlotting factor purified by us was 45.36 kDa. Phospholipase A_2, the prime agent for anticlotting had a molecular weight of 18 kDa, but larger molecular weight anticlotting factors have been obtained from the venom of *Crotalus atrox*, which contained two compounds of 60 kDa and 21.5 kDa. Also, two fibrinogenolytic enzymes of 21.5 kDa and 23.4 kDa from venoms of *Trimersurus mucrosquamaturs* and a fibrinolytic enzyme of 25 and 26 kDa from the venom of *Agkistrodon acutus*[21] have been purified. In conclusion, though much more work needs to be done on this anticlotting factor purified from *Naja naja oxiana*, this glycoprotein is the first of its kind to show such activity in the venom of the Iranian cobra.

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